THE MOLECULAR EFFECTS OF ASPIRIN ON NFκB IN COLORECTAL CANCER

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Dedication

I dedicate this thesis to my parents and my brothers for their guidance and love.

Declaration

I declare that this thesis was composed entirely by myself and that the research presented is my own unless otherwise stated.

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Abstract

Colorectal cancer (CRC) is the most common fatal malignancy in the non-smoking population and Scotland is a high incidence country with an average number of 3445 annual registrations (1997-2001). Despite research and development in clinical practice, the overall 5-year survival is only 51%. Curative surgical resection can increase survival to 70%, but this is only applicable to patients with localised disease. One approach that holds promise in reducing overall disease mortality involves primary prevention using chemopreventive agents. Substantial evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) protect against colorectal cancer, but the molecular basis is not fully elucidated. The host laboratory has previously demonstrated that aspirin-induced apoptosis in colorectal cancer cells is associated with modulation of the NFκB signalling pathway. The transcription factor NFκB regulates expression of genes involved in proliferation, apoptosis and carcinogenesis. Aspirin-induced apoptosis in colorectal cancer cells was found to be dependent on aspirin-induced degradation of IκBα, the NFκB inhibitory protein, and NFκB nuclear translocation. Epidemiological data indicates that the protective effect of NSAIDs is greater in colorectal cancer compared to other cancer types, suggesting the possibility that aspirin might target distinct molecular pathways in colonic epithelial cells.

The aims of this thesis were to investigate the specificity of this response for colorectal cancer compared to other cancer cell types and to identify any potential molecular markers for the response; to determine the influence of mismatch repair (MMR) and p53

status on the NFκB apoptotic response; to examine whether any detectable modulation of NFκB signalling occurs within the constrained environment of clinical studies and finally to analyse NFκB pathway genes *Rel A* and *IkBa* for mutations in colorectal cancer.

To investigate the question of specificity, the effects of aspirin on cell viability and NFκB signalling were studied in a panel of colorectal cancer cell lines compared to cancer cell lines of non-colonic origin. Aspirin induced a concentration-dependent decrease in viable cell number, paralleled by proportionate increases in apoptosis in six colorectal cancer cell lines. There was no consistent change in apoptosis in the five non-colorectal cancer cell lines studied. In colorectal cancer cell lines, aspirin-mediated apoptosis was associated with aspirin-induced IκBα degradation and NFκB nuclear translocation. In contrast, these changes in NFκB signalling were absent in non-colorectal cancer cell lines, paralleling the lack of changes in cell viability and apoptosis observed. This work establishes the degree of specificity of the aspirin-induced NFκB apoptotic response in colorectal cancer. Further work to elucidate the molecular mechanism of this differential sensitivity may lend insight into the mode of action of NSAIDs, and identify molecular markers of response. Basal expression levels of COX-2 or of β-catenin did not relate to susceptibility to NSAID-induced apoptosis. This suggests that COX-2 and β-catenin are unlikely to play a predominant role in aspirin-mediated apoptosis via the NFκB pathway. However, this work does emphasise the generality of the aspirin-induced NFκB apoptotic response with respect to colorectal cancer genotype.

The influence of MMR and p53 status on the NFκB apoptotic response was determined, as these pathways are deranged in colorectal cancer, and are implicated as NSAID targets. Furthermore, this may potentially identify which subsets of colorectal cancer, with respect to genotype, are susceptible to aspirin-mediated chemoprevention. This was investigated by studying the effects of aspirin on HCT-116 (MMR deficient & wild-type p53), HCT 116^{+ch3} (MMR proficient & wild-type p53) and HCT-116 $^{p53-/-}$ (p53 null) colorectal cancer lines. Aspirin induced a dose-dependent decrease in viable cell number, paralleled by proportionate increases in apoptosis in all three cell lines, which was accompanied by IκBα degradation and NFκB nuclear translocation in each case. These results show that the aspirin-induced NFκB apoptotic response occurs irrespective of MMR and p53 status.

It is important to investigate the *in vivo* relevance of these molecular observations. Hence, the effects of aspirin and the COX-2 selective inhibitor rofecoxib were studied in normal mucosa and cancer of rectal cancer patients, and in normal mucosa of genetically predisposed patients before and after ingestion for 7 days. The preliminary results show that there was wide variability, in terms of IκBα expression, between patients and no consistent change was demonstrated between baseline IκBα protein expression and post-treatment levels, in normal mucosa and rectal cancers of patients treated with NSAIDs.

The NFκB pathway plays a central role in death signalling and since altered regulation of NFκB has been observed in colorectal cancers, it is possible that deranged NFκB signalling in colorectal cancer may be due to mutations in the NFκB pathway genes, *Rel A* and *IkBa.* Polymorphic DNA sequence variations, or mutations, in *Rel A* or *IkBa* genes

could account for the variability in NFκB response observed in colorectal cancer patients and also the cell-type specific nature of the NFκB apoptotic response. Hence, mutation analysis of *Rel A* and *IkBa* genes was performed. This has identified a number of interesting variants that merit further investigation.

The work in this thesis underscores the importance of NFκB as a key target for the anti-tumour activity of NSAIDs in colorectal cancer. The results provide evidence of a molecular rationale for the greater specificity of NSAID-mediated protection observed in colorectal cancer compared to other cancers. Furthermore, the lack of association with COX-2 and β-catenin expression and independence from MMR and p53 mutation status emphasises the generality of the aspirin-induced NFκB apoptotic response in colorectal cancer. This is clinically relevant when considering NSAIDs for chemoprevention in genetically predisposed individuals and as adjuvant therapy for sporadic colorectal cancers. The clinical studies demonstrate, for the first time, that changes in IκBα protein levels are detectable in normal mucosa and tumour biopsies from patients. However, the intra- and inter-patient variability in IκBα levels before and after NSAID treatment precludes any conclusion pending further experimentation. The variability requires more detailed controls than the pre- and post- sampling protocols shown here in pilot studies. Overall, this thesis contributes to the understanding of NSAID-mediated apoptosis via modulation of NFκB signalling in colorectal cancer, and may inform chemoprevention trials and novel drug design targeting the signalling pathways involved.

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Abbreviations

ACF-aberrant crypt foci

APC-adenomatous polyposis coli

ARD-ankyrin repeat domain

BMI-body mass index

CLASS -celecoxib long-term arthritis safety study

cIAPs-cellular inhibitors of apoptosis

CRC-colorectal cancer

CTNNB1- β-catenin gene

COX-cyclooxygenase

EMSA-electrophoretic mobility shift assays

FCS- foetal calf serum

FOBT- faecal occult blood testing

FAP-familial adenomatous polyposis

H&E-haemotoxylin and eosin

HEPES-N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HLA- human leukocyte antigen

HNPCC- hereditary non-polyposis colorectal cancer

HRT-hormone replacement therapy

IKK-IκB kinase

IKKβ-IκB kinase β

IκB- inhibitor of NFκB

LOX-lipoxygenase

LOH-loss of heterozygosity

MAP-MYH-associated polyposis

MHRA-Medical and healthcare products regulatory agency

MIN-multiple intestinal neoplasia

MMP-9- matrix metalloproteinase-9

MMR-mismatch repair

MSI-microsatellite instability

MTAP-multi-targeted DNA-based assay panel

MTHFR-methylenetetrahydrofolate reductase

NAG-1-NSAID-activated gene 1

NAT-*N*-acetyltransferase

NLS- nuclear localisation signal

NSAIDs- non-steroidal anti-inflammatory drugs

PI3K-phosphatidylinositol-3-OH kinase

RCT-randomised controlled trials

RHD-rel homology domain

SMAC/Diablo - second mitochondrial-derived activator of caspase / direct inhibitor of apoptosis protein-binding protein with low pI

SRD-signal receiving domain

TAD- transactivation domain

TNFα- tumor necrosis factor α

VEGF -vascular endothelial growth factor

VIGOR-vioxx gastrointestinal outcomes research

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Chapter 1

Introduction

1.1 Colorectal Cancer

1.1.1 Incidence- demographic data, trends and survival

Colorectal cancer is the third most common cause of cancer with 1 million new cases annually, comprising 9.4% of total cancer burden world wide (Parkin *et al.*, 2005). The disease is also ranked third as a cause of cancer mortality with 529 000 deaths per annum worldwide. The gender distribution is similar for colon cancer (M: F, 1.1:1) but is slightly greater in males for rectal cancer (M: F, 2:1). The rectal cancer risk is in fact 50% greater in males, but there are fewer males alive in the peak age range. There is at least a 25-fold variation in incidence between countries, which is high in North America, Western Europe, Australia, and low in Africa and Asia (Parkin *et al.*, 2005). The incidence has been increasing in countries where the risk of colorectal cancer is low, such as parts of Asia and Eastern Europe but has not changed in the high-risk countries (Parkin *et al.*, 2005). The rise in colorectal cancer incidence in low risk countries has been attributed to progressive 'Westernisation' of lifestyle including diet. In contrast, the relative stabilisation of incidence in high-risk countries may be due to improved health awareness in younger generations. Scotland is a high incidence country, with an average number of 3445 annual registrations (1997-2001), and colorectal cancer is the second commonest

cause of cancer death after lung cancer (Information and Statistics Division, 2005). The lifetime risk of developing colorectal cancer for males is 6.1% and for females is 5% in Scotland (1997-2001) (Information and Statistics Division, 2005). Extrapolation of current trends in Scotland shows that the incidence of colorectal cancer is predicted to increase in males, with a lesser increase in females, and mortality is predicted to decrease over the next decade (Dunlop, 2001). Globally, incidence rates have been increasing in countries where they were previously low and this has been attributed to changes in diet and lifestyle.

1.1.2 Aetiology

Differences in incidence of colorectal cancer observed between developing nations and developed or Western countries indicate a predominantly environmental aetiological component. Data from studies of Japanese migrants to Hawaii show that the incidence of colorectal cancer in first generation migrants rapidly reaches the high incidence rates observed in the indigenous population (Kolonel *et al.*, 2004). The high incidence rate in first generation migrants indicates that risk factors could act relatively late in life, suggesting that environmental causes play a significant role in the initiation of colorectal cancer. In fact, as much as 70% of risk may be attributed to environmental factors and the remaining aetiological contribution is due to genetic susceptibility.

1.1.2.1 Environmental factors

Environmental risk factors are defined as surroundings, conditions or influences that affect tumour growth and these may be divided into dietary and lifestyle characteristics.

The first observations of this nature were made by Burkitt, who suggested that low colorectal cancer incidence in African countries was due to greater intake of fibre compared to Western countries (Burkitt, 1971). Numerous observational studies have indicated a protective effect attributed to fibre consumption, with 60% of studies showing a relative risk of less than 0.8 with the highest fibre intake (Gatof and Ahnen, 2002). Two recent observational studies have shown that dietary fibre intake is associated with decreased risk of distal colon adenomas and colorectal carcinomas (Bingham *et al.*, 2003;Peters *et al.*, 2003). Putative anti-tumour mechanisms include shortened stool transit time and hence exposure to carcinogens. Fibre may also lower stool pH by binding bile acids and thereby block conversion to harmful secondary bile acids. Butyrate is a short-chain fatty acid produced by colonic bacterial-induced fermentation of fibre and has been shown to protect against colorectal cancer depending on the concentration, duration of exposure, and the type of fat in the diet (Lupton, 2004). Butyrate has also been shown to be anti-neoplastic in colorectal cancer cell lines (Coxhead *et al.*, 2005).

There is currently no evidence from randomised controlled trials to indicate that increased dietary fibre intake will reduce the incidence or recurrence of adenomatous polyps within a two to four year period (Fuchs *et al.*, 1999; Schatzkin *et al,* 2000; Alberts *et al.,* 2000; Asano and McLeod, 2002). However, the time-period of these studies was only 3-4 years and it is possible that longer intervention and follow-up may reveal a protective effect, or indeed that the protection is imparted early in life. These studies highlight the drawback of using adenomas as surrogate markers, as the majority of polyps do not progress to cancer. However, it is concerning that there was a high rate of adenomas in both low and high fibre groups in one study (Alberts *et al.*, 2000), and in a phase III trial, fibre

increased the risk for recurrent adenomas by 67% (Bonithon-Kopp *et al.*, 2000). Indeed, an *increased* risk of cancer by 35% was reported in one study (Fuchs *et al.*, 1999).

Diets high in fat, especially animal fat, have been associated with an increased risk of colorectal cancer and about 50% of studies show an increase in the relative risk (> 1.2) with the highest dietary fat intake (Gatof and Ahnen, 2002). The mechanism of action may be related to high fat diets inducing increased bile acid secretion, which can be converted to carcinogens by colonic bacteria. Independent of fat, ingestion of red meat has also been associated with an increased risk of colorectal cancer (Willett *et al.*, 1990; Giovannucci *et al.*, 1994). The carcinogenic constituents are thought to be heterocyclic amines formed during high temperature cooking of meat (Butler *et al.*, 2003). Heterocyclic amines require metabolic activation by *N*-acetyltransferase (NAT) prior to binding DNA and *NAT1* and *NAT2* genes are responsible for activity (Lang *et al.*, 1994). Polymorphisms at the NAT1 or NAT2 locus resulting in rapid acetylation have been shown to increase colorectal cancer risk, exemplifying gene-environment interactions (Welfare *et al.*, 1997; Le Marchand *et al.*, 2001).

There is conflicting data on the role of fruit and vegetables and colorectal cancer risk, with some evidence suggesting a lower risk of colorectal cancer (Trock *et al.*, 1990), but other work indicating no protective effect (Michels *et al.*, 2000). It is also unclear as to which particular micronutrient may be responsible for any protection conferred. Folate is one such micronutrient found in fruit and vegetables that may decrease colorectal cancer risk (Giovannucci *et al.*, 2003; Martinez *et al.*, 2004). Folate is an important source of methyl groups that are required for DNA synthesis, repair and methylation, and hence

may influence mutation rate and gene expression (McKay *et al.*, 2004). Polymorphisms in a key enzyme involved in folate metabolism, methylenetetrahydrofolate reductase (MTHFR), have been associated with decreased colorectal cancer risk (Le Marchand *et al.*, 2005b). However, the relationship between dietary intake, colorectal cancer risk and polymorphic variants is complex (Sharp and Little, 2004). High alcohol consumption has been shown to increase the risk of colorectal adenomas and cancer, in particular rectal cancer, possibly by interfering with folate and methionine metabolism (Bagnardi *et al.*, 2001; Giovannucci *et al.*, 1993). There is little doubt that diet plays a role in colorectal cancer, but identification of the specific anti-carcinogenic dietary components and their relative contribution to the any protective effect is difficult to unravel.

Lifestyle characteristics implicated as risk factors include a lack of physical activity and obesity. Increased physical activity has been inversely associated with colorectal cancer incidence with a magnitude of risk reduction as high as 40-50% (Gatof and Ahnen, 2002). A recent study designed to examine for confounding variables in this association found that it was an independent predictor (Slattery and Potter, 2002). Possible mechanisms underlying anti-tumour activity related to physical activity are increased motility of colonic contents, improved immune function and alterations in prostaglandins, insulin, triglyceride and hormone levels.

High body mass index (BMI) is associated with an increased risk of colon cancer, and the association appears to be stronger for men than for women. This difference could be due to the protective effects of oestrogen on colorectal cancer risk in women, since oestrogen levels increase with obesity in women. Substantial evidence indicates that women using

hormone replacement therapy (HRT) have an approximately 30-40% decreased risk of colorectal cancer and this may explain why the reduction in mortality over the last 20 years has been greater in women (Beral *et al.*, 1999). Interestingly, central obesity appears to increase colorectal cancer risk independently of BMI (Giovannucci *et al.*, 1995). Based on the observations that central obesity and physical inactivity are risk factors for colorectal cancer, it has been suggested that associated insulin resistance and consequent hyperinsulinemia may be the principal underlying risk factor (Giovannucci, 1995; Mathers, 2004; Slattery *et al.*, 2005). Increasing studies report that polymorphisms in components of the insulin-related pathway are associated with susceptibility to colorectal cancer (Slattery *et al.*, 2004a; Le Marchand *et al.*, 2005a). The same group suggest that NSAIDs may modulate colorectal cancer risk via an insulin-related pathway, because of reported associations between polymorphisms in insulin receptor substrate 1 and vitamin D receptor genes, NSAID use and colorectal cancer risk (Slattery *et al.*, 2004b). Since inflammation is thought to lead to insulin resistance, NSAIDs may reduce colorectal cancer by exerting anti-inflammatory effects and decreasing insulin resistance.

An early study suggested that approximately one third of the variation in cancer risk may be due to smoking and dietary factors, and that the majority of cancers in those under the age of 65 years may be potentially avoidable (Doll and Peto, 1981). A recent study examined modifiable risk factors and estimated that 30-50% of colorectal cancer risk and 25-30% distal colonic adenoma risk might be preventable by moderate changes in diet and lifestyle (Platz *et al.*, 2000). Indeed, recent randomised controlled trials have shown that daily ingestion of low doses of aspirin reduces the incidence of colorectal adenomas (Baron *et al.*, 2003; Sandler *et al.*, 2003). Although environmental risk factors are

acquired and hence theoretically preventable, experience in other diseases such as ischaemic heart disease has shown that it is difficult to enforce dietary and lifestyle modifications. In fact, the individual contribution of these risk factors to overall incidence is small and lifestyle modification may only lead to a modest reduction in incidence (Dunlop, 2001). Furthermore, it is increasingly apparent that it is the complex relationship between diet and individual genotype that governs colorectal cancer risk (Mathers, 2004).

1.1.2.2 Genetic susceptibility

Genetic determinants increase susceptibility to environmental exposures, and conversely the rate of mutations can be affected by environmental factors. Environmental risk factors may contribute to genetic defects in sporadic colorectal cancer by inducing DNA damage in somatic cells and by formation of carcinogen-DNA adducts resulting in DNA mutations (Gertig and Hunter, 1997). Furthermore, polymorphisms or mutations in enzymes or other metabolic pathway machinery may influence susceptibility to environmental risk factors.

The strongest evidence of a substantial genetic component comes from a study examining 44,788 pairs of twins which indicated that 35% of all colorectal cancer has a heritable component, that is the proportion of susceptibility to cancer accounted for by genetic factors (Lichtenstein *et al.*, 2000). However, less than 5% of colorectal cancers are due to dominant syndromes, such familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), for which mutations in cancer susceptibility genes have been identified (de la Chapelle, 2004). This indicates that there is a substantial genetic contribution to colorectal cancer yet to be elucidated.

Familial adenomatous polyposis is due to dominantly inherited mutations in the adenomatous polyposis coli (*APC*) gene located on chromosome 5q 21. The *APC* gene is a tumour suppressor gene involved in cell adhesion, cell signalling via the *Wnt*/ β catenin pathway and cell proliferation. In addition to the inherited germline *APC* mutation, a somatic mutation in the wild-type allele is required to initiate tumour development (Ichii *et al.*, 1992; Levy *et al.*, 1994). Phenotypically, FAP is characterised by the development of hundreds of colorectal adenomas during the second or third decade of life with 100% risk of developing colorectal cancer if left untreated, as 1 or more adenomas may progress to colorectal cancer (Debinski *et al.*, 1996). FAP patients may also develop extra-colonic manifestations such as retinal lesions, osteomas, desmoids and brain tumours. Disease phenotype has been shown to correlate with the site of mutation within the *APC* gene (Nugent *et al.*, 1994). However, there is considerable phenotypic variation that is not attributable to the site of *APC* mutation and this may be explained by modifier genes (Crabtree *et al.*, 2002). Patients with multiple or early-onset tumours, without mutations in *APC* or *MYH,* do not appear to harbour mutations in other components of the *Wnt* pathway such as β-catenin, *APC*2, axin, and glycogen synthase kinase-3β (Lipton *et al.*, 2003). In sporadic colorectal neoplasia, *APC* inactivation has also been demonstrated in aberrant crypt foci, adenomas and in the majority of carcinomas (Powell *et al.*, 1992; Jen *et al.*, 1994). The detection of *APC* mutations in the early stages of colorectal neoplasia indicates the critical role of *APC* in colorectal cancer initiation (Fodde *et al.*, 2001).

Hereditary non-polyposis colorectal cancer is a dominantly inherited syndrome accounting for 1-5% of all colorectal cancer. HNPCC is due to mutations in the DNA mismatch repair system, which serves to eliminate mismatch errors during DNA

replication. In contrast to FAP, there are no clinical features that are pathognomonic of HNPCC but it is characterised by early-onset cancer, fewer adenomas and extra-colonic cancers such as endometrial, ovarian, stomach, small intestine and uroepithelial. Empiric criteria for diagnosis of HNPCC are three or more relatives with histologically proven colorectal cancer, one being a first-degree relative of the other two; two or more generations affected; and at least one family member affected before age 50 years (Vasen *et al.*, 1991; Vasen *et al.*, 1999). Nine MMR repair genes have been identified but 70-85% of mutations affect *hMLH1* and *hMSH2* and the remainder are in *hPMS2* and *hMSH6* (Lynch and de la, 2003). DNA from HNPCC cancers displays a characteristic instability, microsatellite instability (MSI), due to replication errors within mononucleotide repeats. Hence, genes with repetitive DNA sequence such as *BAX*, *TGFβRII* and *CTNNB1* may accumulate mutations as a result of replication errors. Interestingly, about 13-15% of sporadic colorectal cancers exhibit MSI (Boland *et al.*, 1998; Brown *et al.*, 1998). However, in sporadic cancers MSI is due to promoter hypermethylation of *hMLH1* rather than somatic mutations found in HNPCC (Kuismanen *et al.*, 2000). Defects in the MMR system result in a mutation rate, which is 2-3 times greater than normal cells culminating in accumulation of mutations and tumour progression (Bhattacharyya *et al.*, 1994).

Mutations in other genes have been described resulting in syndromes that predispose to colorectal cancer, but these constitute a minority of the total disease burden. All such syndromes are characterised by the presence of benign adenomas. Peutz-Jeghers is a dominantly inherited syndrome characterised primarily by predisposition to benign intestinal hamartomatous polyps and is due to inactivating mutations in the protein kinase

gene *LKB1* (*STK11*) (Hemminki *et al.*, 1998). Juvenile polyposis is dominantly inherited with mutations in *SMAD4* and more recently *BMPR1A* genes, reflecting the genetic heterogeneity of this syndrome (Lynch *et al.*, 1997; Howe *et al.*, 1998; Howe *et al.*, 2001a). Mutations in *PTEN,* a phosphatase that negatively regulates the phosphoinositol-3-kinase (PI3K)/Akt pathway and mediates cell-cycle arrest and apoptosis, are responsible for the dominantly-inherited Cowden's disease which is characterised by colonic hamartomatous polyps (Liaw *et al.*, 1997). However, the majority of genetically determined colorectal cancers are not due to highly penetrant single gene mutations. MYH-associated polyposis (MAP) is an autosomal recessive syndrome due to bi-allelic mutations in the base excision repair gene *MYH* (Al Tassan *et al.*, 2002). Phenotypically, MYH-associated polyposis is similar to attenuated FAP. Germline *MYH* mutation carriers have been shown to be associated with an increased risk of colorectal cancer (Halford *et al.*, 2003; Croitoru *et al.*, 2004). The *MYH* gene has been localised to 1p32-34 (Slupska *et al.*, 1996). Both pre-malignant lesions and colorectal cancers display loss of heterozygosity (LOH) at 1p, and tumours with 1p LOH harbour monoallelic *MYH* variants (Kambara *et al.*, 2004). There is accumulating evidence supporting the presence of low-penetrance variants as colorectal cancer susceptibility alleles, and such variants may be responsible for the multifactorial inheritance of colorectal cancer (Kemp *et al.*, 2004).

In addition to recognised mutations that account for familial colorectal cancer syndromes, the genetic basis for sporadic colorectal cancer is well established as a multi-step model of carcinogenesis (Fearon and Vogelstein, 1990) (Figure 1.1). Colorectal cancer results from the mutational activation of oncogenes and the inactivation of tumour suppressor

genes resulting in an accumulation of genetic aberrations, each conferring a selective growth advantage promoting clonal tumour development. There are at least seven independent genetic events required for malignant transformation (Kinzler and Vogelstein, 1996) and the cumulative effect of these somatic mutations results in colorectal cancer. There are sequential changes that are required for colorectal cancer development in four genes: *APC, KRAS, SMAD4* and *p53* (Fodde *et al.*, 2001). The sequential order of these genetic changes appears to be important in determining the biological behaviour of tumours. *APC* mutations are evident in the earliest form of neoplastic development such as aberrant crypt foci and are responsible for initiation of tumorigenesis. Additional mutations such as in *KRAS*, which are present in 50% of colorectal cancers, result in tumour progression. Subsequent mutations in *SMAD4* and *p53* occur later in the tumorigenic sequence promoting further clonal expansion and malignant transformation.

It has been suggested that research focus should be on molecular and signalling pathways rather than individual genes, given that mutations in several different genes in the same pathway can lead to cancer (Vogelstein, 2006). For example, mutations in *APC* and β-catenin have been found in 85% and 8% of sporadic colorectal cancers respectively, but there were no tumours with mutations in both *APC* and β-catenin. In fact, Vogelstein *et al* have shown that all the gene pairs in Figure 1.1 are mutually exclusive in terms of mutations, and suggest that approximately two-thirds of all sporadic colorectal cancers have mutations in each of these pathways. In FAP, patients have numerous adenomas and cancer develops as a result of one or more of these progressing as other mutations are acquired, whereas in HNPCC the rate of accumulation of mutations is accelerated.

Although the model explains the development of both familial and sporadic colorectal cancers to some extent, there is a large hereditary component of colorectal cancer that is as yet to be elucidated.

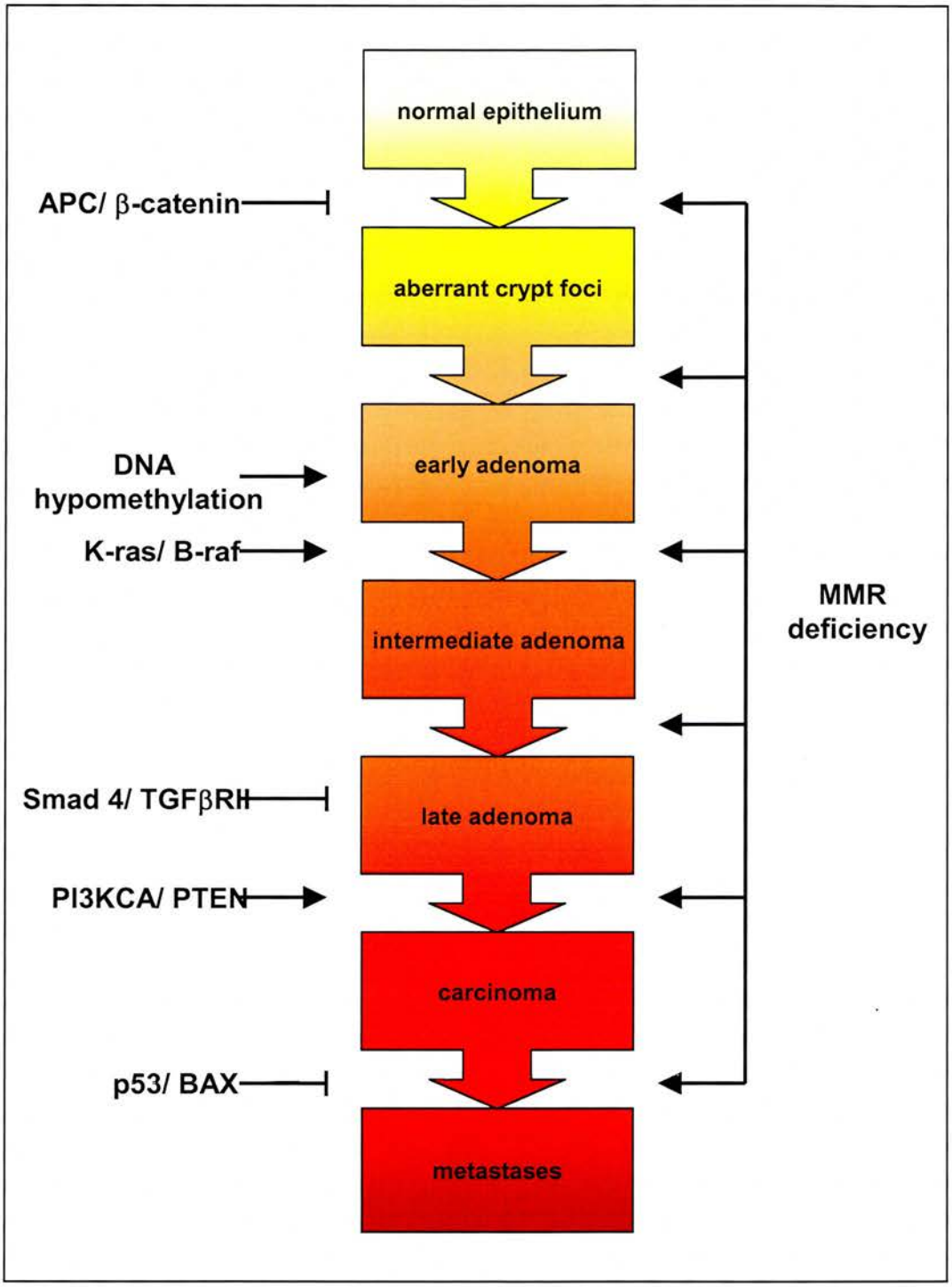

Figure 1.1 Genetic changes associated with the adenoma to carcinoma sequence in colorectal cancer. Development of colorectal cancer is due to oncogene activation and inactivation of tumour suppressor genes resulting in an accumulation of genetic alterations. Genetic changes correlate with histological transformation from normal mucosa to carcinoma. Gene pairs have been shown to be mutually exclusive in terms of mutations. *Adapted from Vogelstein 2006 (AACR Webcast)*

1.1.3 Treatment of Colorectal Cancer

Despite continuing research and development in both basic sciences and clinical practice, the overall 5-year survival from colorectal cancer remains low at 51% (Information and Statistics Division, 2005). Although mortality from colorectal cancer has decreased over the last 40 years, this is primarily due to progress in surgical technique and peri-operative care rather than any intervention that actually alters the natural history of the disease. Surgery remains the mainstay of treatment for colorectal cancer, and offers the best chance of a cure with 5-year survival following curative resection being 75% (Arbman *et al.*, 1995; Gordon *et al.*, 1993; Kanemitsu *et al.*, 2003; Frizelle *et al.*, 2002). Although curative surgery can increase survival, this is only appropriate in patients presenting with localised disease. Advanced disease is present in approximately 20-24% of colorectal cancers at presentation (Mella *et al.*, 1997; Smith *et al.*, 2003). Whether curative or palliative, 90% of patients require surgery and there is considerable disease-related morbidity and mortality.

Currently, the most accurate predictor of overall survival is stage of the cancer at presentation. Colorectal cancer is staged by the TNM classification, which describes the extent of the primary tumour (T), the involvement of regional lymph nodes (N), and the absence or presence of distant metastasis (M). Colorectal cancer also continues to be staged using the Dukes' classification (Dukes, 1951). Dukes' A tumours do not extend beyond the muscuaris propria, Dukes' B tumours extend into the subserosa and serosa and Dukes' C tumours represent lymph node metastasis. The 5-year survival for Dukes' A, B and C is 71-85%, 57-64% and 36-38% respectively (Mulcahy and O'Donoghue, 1997). The overall 5-year survival for advanced colorectal cancer, which constitutes a

significant proportion of the disease, remains poor at 3-10% (Mulcahy and O'Donoghue, 1997). The majority of colorectal cancer presents with later stage disease, and the distribution by stage at presentation is 13% with Dukes A, 37% with Dukes B, 22% with Dukes C and 24% with Dukes D (Smith *et al.*, 2003). Clearly, both a curative outcome and improved 5-year survival are more likely if colorectal cancer is detected at an earlier stage. Colorectal cancer treatment is tailored for different stage disease, but surgical resection remains the most effective. In addition to predicting survival, Dukes' staging is also used to indicate those who require adjuvant therapy such as chemotherapy and radiotherapy.

An absolute survival benefit of 4-13% with adjuvant chemotherapy has been demonstrated in Dukes C colon cancers, with weaker evidence for Dukes C rectal cancers (IMPACT, 1995; Dube *et al.*, 1997). Hence, patients with Dukes C colorectal cancers should be considered for adjuvant chemotherapy. The evidence is less convincing for Dukes B tumours (IMPACT, 1995; Figueredo *et al.*, 1997; Figueredo *et al.*, 2004). The QUASAR 1 trial randomised patients between adjuvant chemotherapy and observation and demonstrated a small (1-5%) survival benefit in Dukes B patients (Gray *et al.*, 2004). Hence, given the potential toxicity routine use is not currently justified for patients with Dukes B tumours. Such patients may be offered chemotherapy in the context of trials, or if considered to have a 'high-risk' tumour exhibiting features such as lymphovascular invasion and poor differentiation (IMPACT, 1995).

It is clear that adjuvant radiotherapy significantly decreases local recurrence following curative resection of rectal cancer. The Dutch trial demonstrated a significant decrease in

local recurrence from 8.2 to 2.4% with short-course preoperative radiotherapy, compared to total mesorectal excision alone (Kapiteijn *et al.*, 2001). However, no significant survival benefit was demonstrated in a recent meta-analysis (Colorectal Cancer Collaborative, 2001), which contradicted both the Swedish Rectal Cancer Trial (Swedish Rectal, 1997) and a previous meta-analysis (Camma *et al.*, 2000). Pre-operative radiotherapy does lead to increased sexual dysfunction, slower recovery of bowel function, and impaired daily activity post-operatively (Marijnen *et al.*, 2005). Nonetheless, a decrease has been demonstrated in local recurrence, which can be painful and incapacitating. Hence, a selective approach is adopted by most, and pre-operative radiotherapy is used for fixed or tethered T3/4 tumours and very low tumours in relation to the anal sphincter. Post-operative radiotherapy is less beneficial, in terms of local recurrence, than pre-operative radiotherapy and has no effect on survival, and thus is reserved for those with positive resection margins. Pre-operative combined chemoradiation may be used to downstage disease to allow sphincter preserving surgery (Habr-Gama *et al.*, 2004) and has been shown to confer a small survival benefit (Chau *et al.*, 2003; Gunderson *et al.*, 2003). The survival benefit has to be balanced against toxicity-related morbidity and impact on quality of remaining life.

Despite most patients undergoing potentially curative surgery and the availability of adjuvant chemotherapy, about 50% of patients presenting with colorectal cancer die from subsequent metastatic disease and 30-50% of patients treated with curative surgery will develop advanced disease (Griffin *et al.*, 1987; Abulafi and Williams, 1994; Young and Rea, 2000). The annual expenditure on colorectal cancer is approximately £230 million in the United Kingdom. This estimate may not include the cost of investigating symptomatic patients to exclude the diagnosis of cancer, and so the true expenditure is probably far

greater. There is no doubt as to the considerable advances in chemotherapy, and yet no agent has been developed which imparts a significant survival benefit in colorectal cancer. It is clear that survival may be improved by earlier detection or by strategies aimed at prevention of the disease.

1.1.4 Screening and early detection

In view of the proportion of patients who present with advanced disease, there is substantial rationale for colorectal cancer screening. Early detection of colorectal cancer would lead to a shift in stage of presentation and thus impact upon survival. Colorectal cancer biology is well characterised in terms of the adenoma-carcinoma sequence, and hence lends itself to early detection. The incidence of adenomas from colonoscopy studies in developed countries has shown rates ranging from 25 to 41% (Markowitz and Winawer, 1997). However, given that the incidence of colorectal cancer is lower, only 10% of adenomas will progress and there are no means of predicting which adenomas will develop into cancer. In addition, recent data from The National Polyp Study suggest that adenoma prevalence results from a dynamic process of both formation as well as regression of adenomas, which has implications for screening (Loeve *et al.*, 2004). Nonetheless, removal of adenomas by endoscopic polypectomy has been shown to decrease the incidence of colorectal cancer (Citarda *et al.*, 2001). The duration between development from adenoma to carcinoma has been shown to be 5 to 10 years (Winawer *et al.*, 1997), which provides a 'latent period' for screening and subsequent intervention.

Several potential modalities have been investigated for the purposes of screening including barium enema, colonoscopy, flexible sigmoidoscopy and faecal occult blood testing (FOBT). Barium enema is not as sensitive in detection of small adenomas since the reported rate of detection of all adenomas was only 39% and only 48% of adenomas > 1cm were detected (Winawer *et al.*, 2000). A 60-95% reduction in mortality from colorectal cancer *in the area examined* was demonstrated with rigid sigmoidoscopy (Newcomb *et al.*, 1992; Selby *et al.*, 1992). Flexible sigmoidoscopy is currently being evaluated as a potential screening tool (Atkin *et al.*, 1998). However, the obvious disadvantage is the incidence of proximal colon cancers and adenomas in people with a normal sigmoidoscopy (Rex *et al.*, 1999; Imperiale *et al.*, 2000). Colonoscopy has the advantage of visualising the whole colon, and polypectomy results in a reduction in colorectal cancer incidence (Citarda *et al.*, 2001). However, there is a definite morbidity and mortality risk associated with colonoscopy, which has to be balanced against potential benefit. Hence, non-invasive screening modalities may be preferable. CT colonography is emerging as a potential screening investigation but there are concerns regarding the sensitivity of the test especially for small polyps and flat lesions (Mulhall *et al.*, 2005; Park *et al.*, 2005). Colonoscopy still had the highest sensitivity for detecting colonic polyps and cancers in a prospective comparison with barium enema and CT colonography (Rockey *et al.*, 2005).

Faecal occult blood testing is being evaluated as a population based screening tool. There have been four randomised controlled trials evaluating FOBT in approximately 350,000 individuals (Kewenter *et al.*, 1994; Hardcastle *et al.*, 1996; Kronborg *et al.*, 1996; Mandel *et al.*, 2000). These have shown that annual FOB testing reduced colorectal cancer

incidence by 20% and colorectal cancer mortality by 16% (Towler *et al.*, 1998). However, sensitivity of the FOB test varied from 46% to 92% and remains an issue especially concerning detection of adenomas that are not bleeding (Towler *et al.*, 1998). In fact, these studies showed a shift in the stage of distribution rather than an actual reduction in colorectal cancer incidence within the study period. The number needed to screen is 1400 to prevent one colorectal cancer death in 5 years (Rembold, 1998). Hence, unnecessary investigation due to the low positive predictive value of FOB testing, with the associated morbidity and mortality, is a key disadvantage. A new stool based test that is currently being developed is the multi-targeted DNA-based assay panel (MTAP), which detects 15 genetic alterations associated with colorectal cancer and has greater sensitivity and specificity that FOBT (Ahlquist *et al.*, 2000).

It is evident that no optimum screening modality has been identified for population-based screening. However, surveillance is justified in high-risk groups such as patients with HNPCC and FAP and in moderate risk patients with a significant family history, where the risk benefit ratio is more favourable. In light of the high incidence of colorectal cancer and the modest improvement in overall mortality, research efforts are being focused not only towards earlier diagnosis and better treatment, but also prevention of the disease using complementary strategies. Chemoprevention is the use of an agent that interrupts, reverses or delays the development of invasive cancer by targeting key molecular aberrations (Sporn, 1976). Amidst several potential agents currently being researched, NSAIDs are conspicuous as the most convincing and consistent chemopreventive agents that appear to be effective at all stages of colorectal cancer ranging from aberrant crypt foci, to adenomas and carcinomas.

1.2 NSAIDs and colorectal cancer prevention

There are several complimentary lines of evidence, from epidemiological and observational studies; clinical trials, animal models and *in vitro* studies that suggest NSAIDs possess anti-tumour activity against colorectal cancer.

1.2.1 Epidemiological evidence

1.2.1.1 Colorectal cancer

Substantial epidemiological evidence indicates that NSAIDs protect against development of colorectal cancer. There is a 40-50% risk reduction in incidence of and mortality from colorectal cancer in both cohort and case-control observational studies (Figure 1.2). Taken together these 29 studies represent more than 18000 cases of colorectal cancer and provide consistent evidence that aspirin and other NSAIDs reduce the overall risk by 40-50%. The reliability of the anti-tumour effect is substantially strengthened by the fact that the reduction in relative risk is consistent despite differing NSAID types and doses, duration of ingestion, study groups and geographical location.

Only two studies have failed to demonstrate a protective effect from NSAID ingestion (Paganini-Hill *et al.*, 1989; Sturmer *et al.*, 1998). The first study investigated the use of NSAIDs in an elderly retirement community (Paganini-Hill *et al.*, 1989). Since NSAID use was determined at the beginning of the study, ingestion may have actually been commenced or discontinued in the ensuing study period. Moreover, there are no data available on the duration of NSAID use. This study also showed an increased risk of ischaemic heart disease in aspirin users indicating the duration of aspirin use may have

been short. The second study that did not demonstrate any reduction in colorectal cancer incidence with NSAID use was the Physicians' Health Study (Gann *et al.*, 1993). This study was prematurely terminated because primary end-points were cardiovascular and there was a significant reduction in myocardial infarction. Furthermore, moderate alcohol intake appeared to be a predictor of aspirin use and this may have confounded the results. The natural history of development from adenoma to carcinoma is ten to fifteen years and it may be that a short duration intervention does not interrupt this sequence of events (Winawer, 1999).

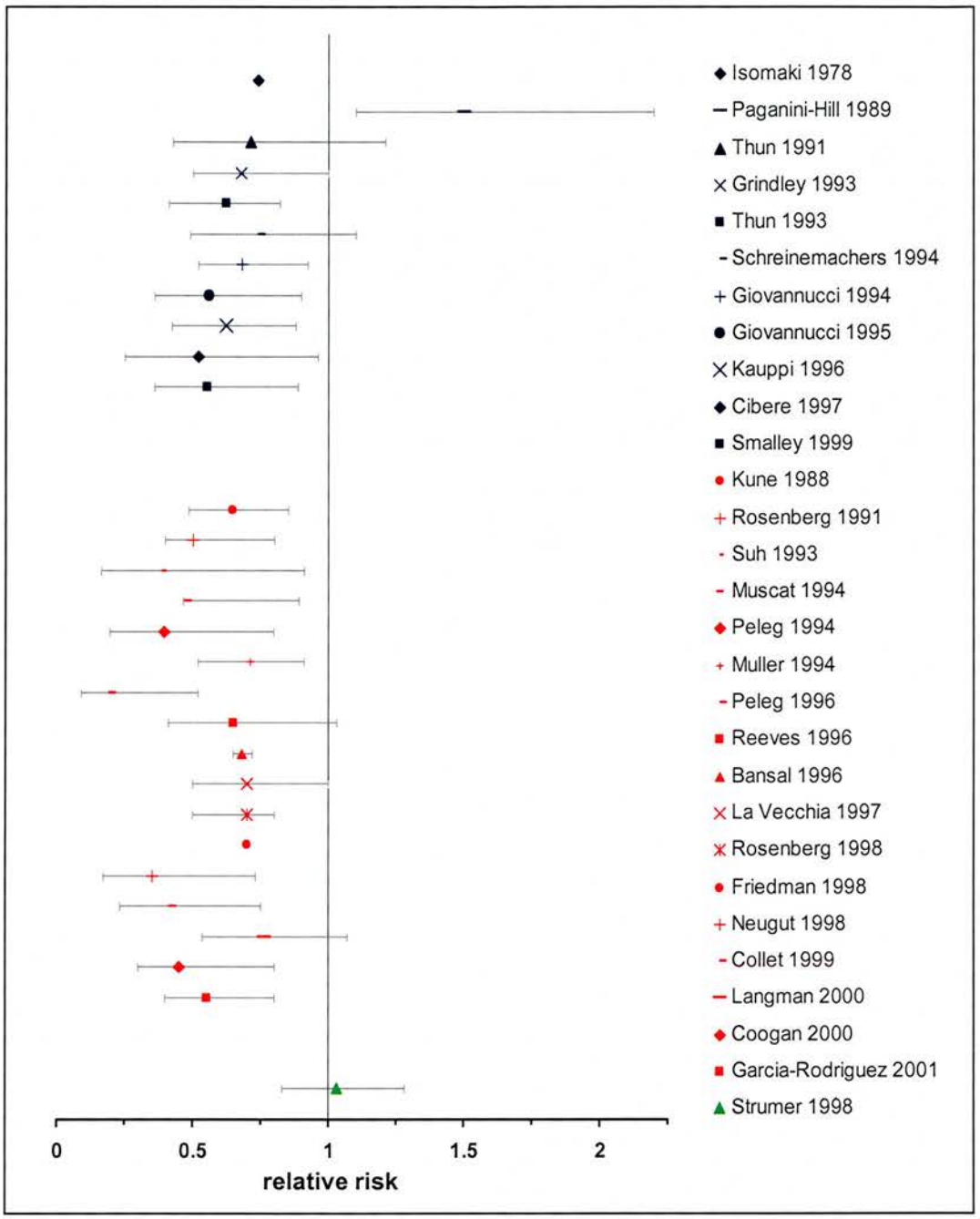

Figure 1.2 Epidemiology studies of association between NSAIDs and colorectal cancer. The relative risk estimates and confidence intervals refer to incidence and death rate among regular NSAID users compared to non-users in cohort (blue) and case-control (red) studies and in randomised controlled trials (green).

1.2.1.2 Sporadic colorectal adenomas

Furthermore, numerous studies have shown NSAID users have a decreased risk of developing colorectal adenomas, indicating that NSAIDs may affect early stages of tumour development (Figure 1.3). These observational studies in isolation provide strong evidence that NSAIDs retard the formation of adenomas. Since it is not feasible to perform trials with colorectal cancer as the primary end-point, colorectal adenomas have been used as biological surrogate markers. In three clinical trials examining the effect of NSAIDs on sporadic adenomas (Hixson *et al.*, 1993; Ladenheim *et al.*, 1995; Matsuhashi *et al.*, 1997), sulindac treatment resulted in polyp regression in only one study (Matsuhashi *et al.*, 1997), but all of these trials examined small populations (Table 1.1).

Table 1.1 Epidemiology studies of association between NSAIDs and sporadic adenomas

Study	Date	Patients	Dosage	Duration	Design	Results
Hixson	1993	7	5 sulindac 400mg 2 piroxicam 20 mg	6 months	clinical trial	no significant reduction in polyp size or number
Landenheim	1995	44 patients previous polyps	Sulindac 300 mg or placebo	4 months	RCT	no significant reduction in polyp size or number
Matsuhashi	1997	15 patients (20 polyps)	sulindac 300 mg	4 months	clinical trial	65% polyps regressed

Three recent randomised controlled trials have consistently demonstrated the chemopreventive effect of aspirin against adenoma formation (Baron *et al.*, 2003; Benamouzig *et al.*, 2003; Sandler *et al.*, 2003). In fact one trial was terminated early because of the significant protective effect of aspirin compared to placebo (Sandler *et al.*, 2003). All three trials showed a striking protective effect with low doses of aspirin ranging from 81mg to 325mg, which are similar to those used in cardiovascular disease prevention. Paradoxically, 81mg aspirin was found to be more protective than the 325mg aspirin, and hence questions remain about the optimal dose.

It has been suggested that gastrointestinal bleeding secondary to NSAID ingestion leads to earlier detection of tumours and is responsible for the apparent protective effect. However, a case-control study compared the risk of developing colorectal cancer in patients on aspirin compared to other anti-coagulants, and found the protective effect was only present in the NSAID-treated group (Muller *et al.*, 1994). Taken together these randomised controlled trials strengthen the evidence that pharmacological doses of aspirin reduce the incidence of colorectal adenomas in a moderate risk population defined as those who have previously had colorectal adenomas or cancer.

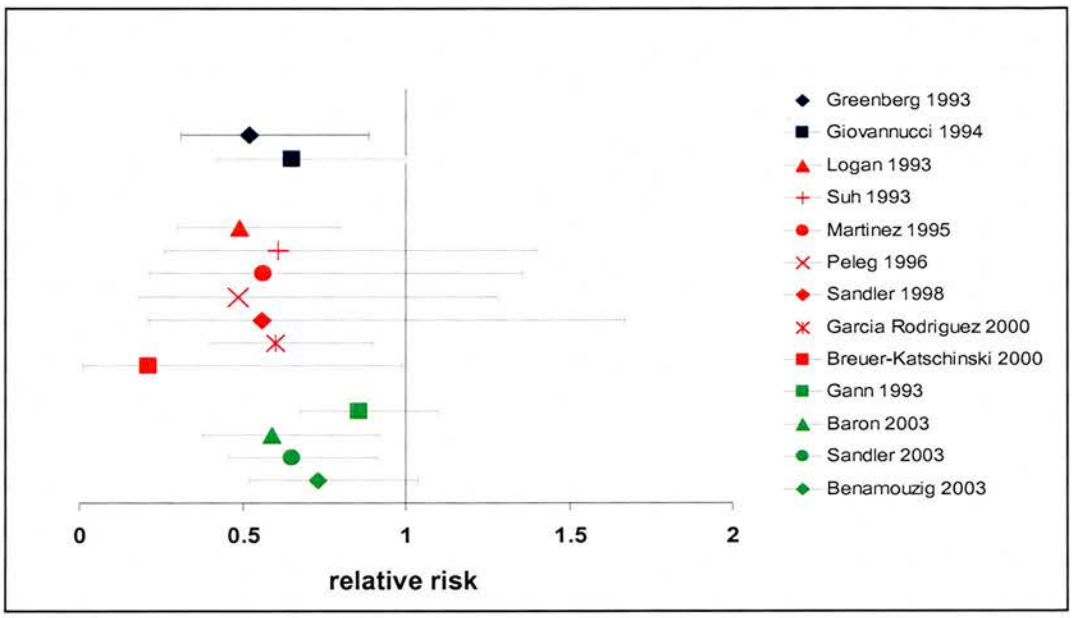

Figure 1.3 Studies of association between NSAIDs and sporadic colorectal adenomas. The relative risk estimates and confidence intervals refer to incidence among regular NSAID users compared to non-users in cohort (blue) and case-control (red) studies and in randomised controlled trials (green).

1.2.1.3 Familial adenoma syndromes

There is also ample evidence that, in addition to sporadic adenomas and colorectal cancers, NSAIDs confer protection in familial syndromes associated with increased risk of colorectal cancer. Indeed, the anti-tumour effect of NSAIDs was initially observed in FAP patients with desmoid tumours, the first of whom was given indomethacin for pericardial inflammation and regression of a mediastinal desmoid was noted (Waddell and Gerner, 1980). Sulindac was then substituted for indomethacin and regression of rectal adenomas was observed in three patients with FAP (Waddell and Loughry, 1983). Several subsequent observational studies have also shown regression of polyps with sulindac treatment (Table 1.2) (Waddell *et al.*, 1989; Rigau *et al.*, 1991; Winde *et al.*, 1993; Spagnesi *et al.*, 1994).

This has been substantiated in three RCTs that demonstrated a reduced number and size of polyps with sulindac (Labayle *et al.*, 1991; Giardiello *et al.*, 1993; Nugent *et al.*, 1993). The largest RCT examining the effect of celecoxib, a selective COX-2 inhibitor, in 77 FAP patients showed 28% fewer polyps and 31% reduction in the size of polyps (Steinbach *et al.*, 2000). Although one study did not show a protective effect with nimesulide, another selective COX-2 inhibitor, on colonic polyps, there was a 15% reduction in duodenal polyps (Dolara *et al.*, 1999).

Table 1.2 Epidemiology studies of association between NSAIDs and familial adenomas

Study	Date	Study Population	Dosage	Duration	Design	Results	p- value
Winde	1993	15	sulindac		trial	66.7% complete remission 33.3%partial remission	
Labayle	1991	9 colectomy & IRA	sulindac 300 mg or placebo	4 months	RCT	66.7% complete remission 33.3%partial remission	<0.01
Nugent	1993	24 duodenal polyps (14 rectal polyps)	sulindac 400 mg OD	6 months	RCT	duodenal polyps < 2mm regressed 9/11 no regression polyps>3 mm	0.01
Giardello	1993	22 (18 not colectomised)	sulindac 300 mg or placebo	9 months	RCT	56% reduction number polyps 65% reduction polyp size	p=0.014 p<0.001
Spagnesi	1994	20 (6 non IRA)	sulindac 200mg	3 months	trial	decrease in number and size of polyps	<0.01
Winde	1997	38 colectomy and IRA	28 sulindac 300mg 10 control	up to 4 years	RCT	78% complete regression 22% partial regression	<.001
Dolara	1999	7	nimesulide 2mg/kg/day	2.5 months	trial	no change in number or size of polyps	
Steinbach	2000	77	32 on celecoxib 200 mg 30 on celecoxib 400 mg 15 on placebo	6 months	RCT	28% reduction number polyps 30.7 % reduction polyp size	p=0.003 p=0.001
Cruz-Correa	2002	12 colectomy & IRA	sulindac mean dose 158mg/day	63 months	RCT	regression of polyp number	0.006

However, there have been concerns regarding the increased incidence of total and non-gastrointestinal serious adverse events, with the COX-2 selective NSAIDs as compared with non-selective NSAIDs, in the Celecoxib Long-term Arthritis Safety Study (CLASS) and the Vioxx Gastrointestinal Outcomes Research (VIGOR) studies (Wright, 2002). Recently, the significantly increased risk of cardiovascular events due to rofecoxib in a polyp prevention trial (APPROVe) led to its withdrawal from the market (Bresalier *et al.*, 2005). The underlying mechanism of action is thought to be preferential COX-2 inhibition resulting in unopposed action of thromboxane leading to thrombotic events and selective renal COX-2 inhibition resulting in salt and water retention, hypertension and exacerbation of cardiac failure (Wright, 2002).

The CAPP-1 study randomised FAP carriers to a placebo or aspirin with or without resistant starch (CAPP-1, 2003). There was no significant reduction due to either intervention in polyp number after a year. However, secondary analysis of data from patients enrolled for more than 1 year showed that both aspirin alone and the combined aspirin/resistant starch treatment resulted in a significant reduction in polyp number. The CAPP-2 study will determine whether aspirin and/or resistant starch reduce adenoma initiation and progression in another genetically predisposed group, HNPCC carriers (CAPP-2, 2005). However, a recent study investigated whether sulindac could prevent adenomas in 41 phenotypically normal FAP patients, and found no difference between the placebo and sulindac group, with respect to both the number and size of polyps (Giardiello *et al.*, 2002). The study period was 4 years and resistance to sulindac has been described which may account for the results. It may also be that the mechanism of action is related to the progression of, rather than the formation of, adenomas or that the doses

used (75 and 150 mg sulindac) were inadequate for primary prevention. However, these doses were sufficient to inhibit prostaglandins, as prostaglandin levels in colorectal mucosa were lower in the sulindac group, which suggests prostaglandin-independent anti-tumour mechanisms may predominate. It has been reported that polyps resume growth in FAP patients once treatment has been stopped and that patients may develop some form of resistance to NSAIDs (Giardiello *et al.*, 1993). There have also been reports of patients developing rectal cancer whilst on sulindac treatment (Niv and Fraser, 1994; Lynch *et al.*, 1995; Giardiello *et al.*, 1996). Nonetheless, these studies provide valuable insights because although FAP itself accounts for only 1-2% of all colorectal cancer, they provide a model of *APC* inactivation which is present in 85% of all colorectal cancers (Thun *et al.*, 2002).

The observation that the protective effect of NSAIDs against both sporadic and familial adenomas is evident with a similar magnitude of risk reduction to carcinomas, suggests that the mechanism of action may be related to an early step in the adenoma-carcinoma sequence. In keeping with this, patients on low doses of aspirin for at least a year showed decreased overall density of aberrant crypt foci, the earliest precursor lesion identified in colorectal cancer development (Shpitz *et al.*, 2003).

In summary, there is overwhelming evidence that NSAIDs protect against development of both colorectal adenomas and cancers in humans with a risk reduction in the order of 30-50%. The striking feature of the epidemiological, observational and RCT evidence is the consistency of the protective effect despite differences in study design, dosage, duration and the type of NSAID used, and the demographics and location of the study populations.

However, there are associated side-effects such as gastrointestinal bleeding which preclude current use of NSAIDs as chemopreventive agents. Hence, there is compelling rationale to understand the molecular mechanism of action as this would not only inform about colorectal cancer biology, but would also permit specific molecular targeting resulting in development of novel chemopreventive agents with increased efficacy and safety.

1.2.2 Animal models

Animal models of carcinogenesis using rodents have been useful for studying the effects of NSAIDs *in vivo*. Colorectal cancers may be chemically-induced by azoxymethane or dimethylhydrazine in rat models, and these also show a reduction in tumour burden in response to treatment with NSAIDs: including sulindac (Piazza *et al.*, 1997a), indomethacin (Pollard and Luckert, 1981; Moorghen *et al.*, 1988; Skinner *et al.*, 1991) nimesulide (Fukutake *et al.*, 1998), pioxicam (Pereira *et al.*, 1996; Piazza *et al.*, 1997a) and a selective COX-2 inhibitor SC58635 (Reddy *et al.*, 1996). Furthermore, NSAIDs decrease the development of pre-neoplastic aberrant crypt foci in azoxymethane rat models (Yoshimi *et al.*, 1997; Shpitz *et al.*, 1998), indicating the mechanism of action may be related to an early stage of tumorigenesis.

The animal model of FAP is the Min (multiple intestinal neoplasia) mouse, which has a mutation in the *Apc* gene and develops small intestinal adenomas. A reduction in tumour burden in Min mice has been demonstrated with several different NSAIDs. The chemopreventive effects occur with both non- COX-2 selective NSAIDs such as sulindac

(Beazer-Barclay *et al.*, 1996; Chiu *et al.*, 1997; Mahmoud *et al.*, 1998a), aspirin (Boolbol *et al.*, 1996; Barnes and Lee, 1998; Mahmoud *et al.*, 1998b), flurbiprofen (Wechter *et al.*, 1997), indomethacin (Chiu *et al.*, 2000) and piroxicam (Jacoby *et al.*, 1996), and also with selective COX-2 inhibitors such as rofecoxib (Oshima *et al.*, 2001).

There are limitations of animal models given that the correlation with human carcinogenesis is imprecise, since Min models develop tumours of the small intestine rather than colon. Nonetheless, animal models have been useful for affording several insights regarding the effects of NSAIDs on specific molecular pathways. The chemopreventive potential of NSAIDs has been shown in more than 90 of the ≥ 100 animal model studies published to date (Hawk ET *et al.*, 2004). Overall, the data indicate that non-selective NSAIDs suppress tumour growth to a greater extent and at lower doses when treatment is begun before or concurrent with carcinogen exposure; that both non-selective and selective NSAIDs inhibit the early stages of carcinogenesis whereas selective COX-2 inhibitors appear to be more effective in later stages and finally NSAIDs must be given continuously to prevent tumour growth (Thun *et al.*, 2002).

1.2.3 Mechanisms of Action

Substantial evidence from epidemiological, animal and *in vitro* work indicates that NSAIDs protect against colorectal cancer, but the molecular basis is not completely understood. Initially, studies have focused upon cyclooxygenase (COX) inhibition and the resultant decrease in prostaglandin synthesis as the basis of NSAID anti-tumour activity, as this is the mechanism underlying their anti-inflammatory activity (Vane, 1971).

However, a growing body of evidence indicates that NSAIDs modulate COX-2 independent pathways, and that COX-2 inhibition may not be the predominant anti-tumour mechanism of action in colorectal cancer (Tegeder *et al.*, 2001). There are several putative anti-tumour mechanisms of action which are COX-dependent and COX-independent: inhibition of cell proliferation, inhibition of carcinogen activation, augmentation of the immune response, inhibition of angiogenesis and the induction of programmed cell death or apoptosis (Figure 1.4).

1.2.3.1 Cox-dependent mechanisms

1.2.3.1.1 Inhibition of carcinogen activation

The anti-tumour activity of NSAIDs may be, in part, due to their ability to inhibit carcinogen activation. Colorectal cancers and adenomas express higher levels of COX-2 compared to normal mucosa (Eberhart *et al.*, 1994). COX enzymes can metabolise pro-carcinogens to carcinogens through their peroxidase activity (Levy, 1997). Examples include the oxidisation of dihydrodiol of benzo[a]pyrene to its epoxide form which can bind DNA and cause mutations, and COX-induced activation of aromatic amines to their mutagenic forms. Prostaglandins can produce malondialdehyde, a mutagen and carcinogen, and can result in the formation of peroxyl radicals, which can activate pro-carcinogens (Shiff and Rigas, 1997). COXs can also indirectly produce free radicals, which can activate transcription factors involved in cell proliferation. Hence, the ability of NSAIDs to attenuate the carcinogen load may contribute to their protective effect in colorectal cancer.

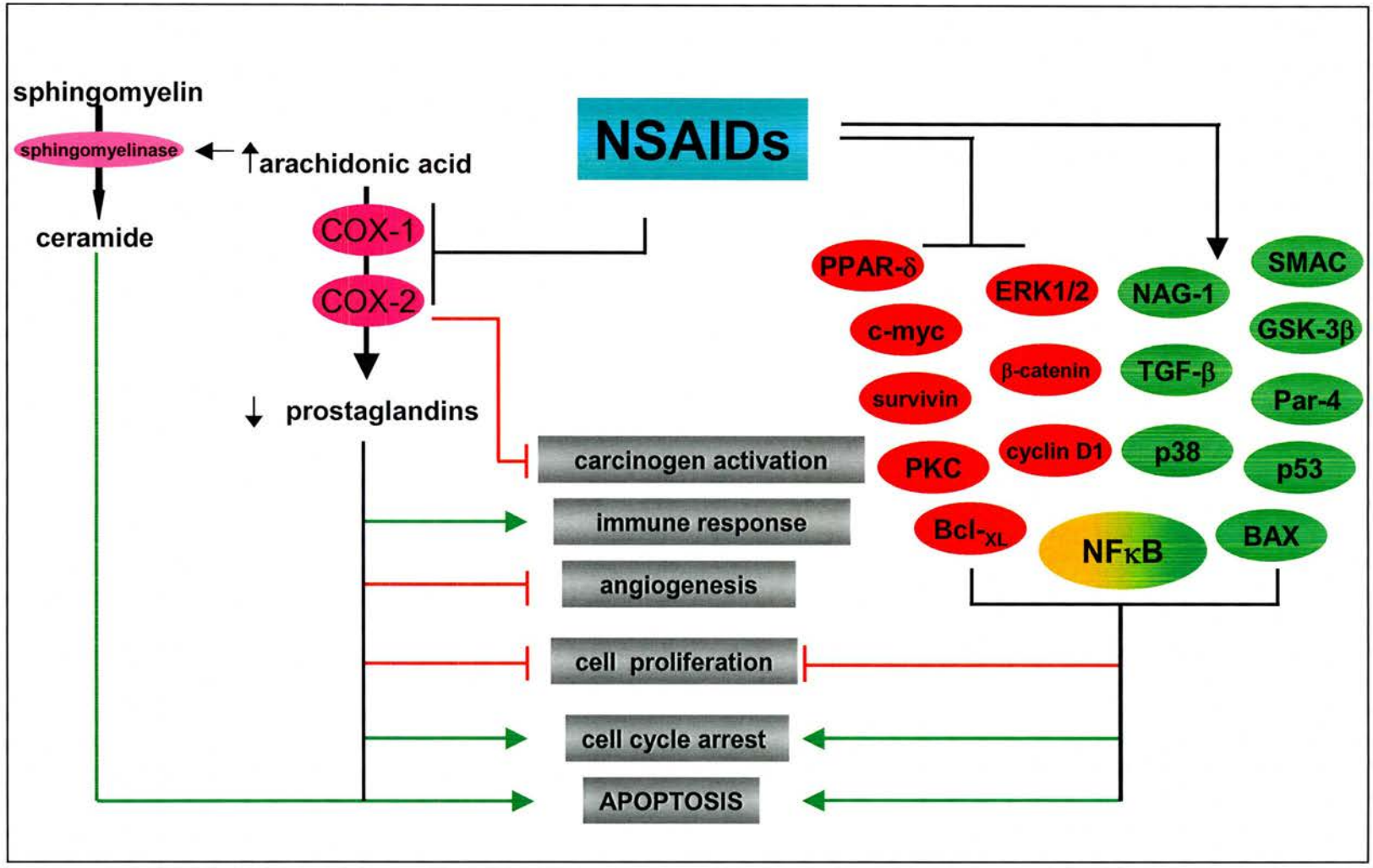

Figure 1.4 Potential COX-dependent and independent mechanisms of NSAID anti-tumour activity. Mechanisms may be COX-dependent or -independent. Target molecules may be inhibited (●), activated (●)or inhibited or activated (●) depending on the stimulus.

1.2.3.1.2 Augmentation of immune response

It is well recognised that tumour cells escape immune-mediated surveillance and subsequent immunologically induced destruction (Ahmad *et al.*, 2004). Human leukocyte antigen (HLA) classes I and II expression is decreased in colorectal cancers and adenomas (McDougall *et al.*, 1990; Tsioulias *et al.*, 1993). Prostaglandins, especially PGE_2, are known to reduce expression of T-cell proliferation and cytotoxicity, macrophage activation and lymphokine production thereby suppressing the immune response (Husain *et al.*, 2002). Therefore, NSAIDs may augment the immune response by inhibiting production of prostaglandins with a resultant increase in expression of HLA genes.

1.2.3.1.3 Inhibition of angiogenesis

Neovascularisation is important for tumour growth beyond 2-3mm in size and subsequent metastasis. Prostaglandin production is increased in colorectal cancer and PGE_2 has been shown to stimulate angiogenesis (Hanahan *et al.*, 1996). Furthermore, COX-1 has been shown to regulate tube formation in endothelial cells (Tsujii *et al.*, 1998), and COX-2 appears to regulate production of angiogenic factors such as vascular endothelial growth factor (VEGF) by colorectal cancer cells (Williams *et al.*, 2000). Indomethacin has been shown to suppress growth of colorectal cancer cells via inhibition of angiogenesis by decreasing VEGF production (Wang and Zhang, 2005). These data suggest that inhibition of angiogenesis by NSAIDs may contribute to their anti-tumour activity.

1.2.3.2 Cox-dependent and independent mechanisms

1.2.3.2.1 Inhibition of cell proliferation

Colorectal cancers have high levels of prostaglandins compared to normal mucosa (Rigas *et al.*, 1993). Since prostaglandins have been shown to stimulate cell proliferation (Qiao *et al.*, 1995), inhibition of prostaglandin production may decrease cell proliferation. Indeed, several cell culture studies have demonstrated that NSAIDs consistently decrease proliferation of colorectal cancer cell lines by inducing cell cycle arrest (Shiff *et al.*, 1995; Goldberg *et al.*, 1996; Shiff *et al.*, 1996; Qiao *et al.*, 1998). Sulindac and rofecoxib, a COX-2 selective NSAID, decrease DNA replication in polyps from $Apc^{\Delta 716}$ Min mice indicating inhibition of cellular proliferation occurs *in vivo* (Oshima *et al.*, 2001). Furthermore, sulindac decreased proliferation indices in rectal mucosa of FAP patients after 33 months of treatment (Winde *et al.*, 1995). However, in another study of sulindac in FAP patients there was no effect on proliferation, but an increase in apoptosis was observed after 3 months (Pasricha *et al.*, 1995). Nonetheless, sulindac has been shown to decrease proliferation in colorectal cancer cells that do not produce prostaglandins because of COX deficiency (Hanif *et al.*, 1996), indicating that prostaglandin inhibition may not be the critical mechanism of action. Furthermore, the concentration of NSAIDs required for COX inhibition are lower than those needed for the anti-tumour effects in cells and animal studies. Although evidence from cell culture, animal and patient studies indicates that NSAIDs are growth inhibitory, it is unclear whether this effect is solely due to decreased prostaglandin production, and hence COX-dependent.

1.2.3.2.2 Induction of apoptosis

Programmed cell death or apoptosis is progressively inhibited during colorectal carcinogenesis (Bedi *et al.*, 1995). Numerous *in vitro* studies have shown that aspirin and other NSAIDs induce apoptosis in colorectal cancer cells (Shiff *et al.*, 1995; Hanif *et al.*, 1996; Shiff *et al.*, 1996; Elder *et al.*, 1997; Piazza *et al.*, 1997b; Qiao *et al.*, 1998; Castano *et al.*, 1999; Stark *et al.*, 2001b). Several putative modes of action, both COX-dependent and COX-independent, have been investigated (Figure 1.4). It has been shown that NSAID-mediated apoptosis may be due to an increase in the prostaglandin precursor arachidonic acid (Chan *et al.*, 1998). The accumulation of arachidonic acid leads to the conversion of sphingomyelin to ceramide, which can induce apoptosis (Jarvis *et al.*, 1996). Arachidonic acid can be metabolised by cyclooxygenases or lipoxygenase (LOX). Inhibition of LOX has also been shown to induce apoptosis and both NS-398 (COX-2 selective) and sulindac lead to upregulation of 15- lipoxygenase-1 (15-LOX-1) which induces apoptosis in colorectal cancer cells (Shureiqi *et al.*, 2000). It has recently been shown that induction of 15-LOX-1 expression is involved in NSAID-mediated down-regulation of PPARδ and the resultant induction of apoptosis in colorectal cancer cells (Shureiqi *et al.*, 2003).

The PPAR family of intracellular nuclear hormone receptors have also been investigated as potential NSAID targets. The PPARs function as ligand-dependant activators of transcription (Lemberger *et al.*, 1996). Sulindac has been shown to suppress PPARδ activity and induce apoptosis, suggesting a mechanism by which sulindac counters the effects of *APC* inactivation that lead to increased PPARδ activity (He *et al.*, 1999). However, a more recent study by the same group showed no difference in sensitivity to

sulindac-induced apoptosis between PPARδ$^{+/+}$ and PPARδ$^{-/-}$ cell lines suggesting that PPARδ is not a major contributor to NSAID anti-tumour activity (Park *et al.*, 2001).

NSAIDs have been shown to modulate expression of both pro- and anti-apoptotic genes. NS-398, a selective COX-2 inhibitor, upregulated expression of the pro-apoptotic gene Par-4 and induced apoptosis in colorectal cancer cells (Zhang and DuBois, 2000). Sulindac has been shown to increase expression of the pro-apoptotic gene NAG-1 (NSAID-activated gene 1) in human colorectal cancer cells and in mouse colon (Kim *et al.*, 2002). Survivin is an anti-apoptotic protein that is over expressed in colorectal cancer (Ambrosini *et al.*, 1997). Sulindac decreases survivin expression in colorectal cancer cell lines (Zhang *et al.*, 2004). NSAIDs have been shown to inhibit the expression of the anti-apoptotic protein Bcl-$_{XL}$, resulting in an altered ratio of BAX to Bcl-$_{XL}$ and apoptosis in colorectal cancer cells (Zhang *et al.*, 2000). It has recently been shown that NSAIDs induce cytoplasmic translocation of the second mitochondrial protein to be released during apoptosis, SMAC/Diablo (Kohli *et al.*, 2004). NSAID-induced apoptosis is abrogated when SMAC/Diablo is disrupted by homologous recombination and RNA interference in colorectal cancer cells, suggesting it plays an important role in NSAID-mediated apoptosis. The p38/MAPK signalling pathway may be involved since sodium salicylate induced-apoptosis in colorectal cancer cells was blocked by SB203580, a p38 MAPK inhibitor (Lee *et al.*, 2003). Aspirin has also been shown to phosphorylate GSK-3β, a multifunctional serine/threonine kinase that negatively regulates *Wnt*/β-catenin signalling, in colorectal cancer cell lines (Dihlmann *et al.*, 2003). Additional potential targets in NSAID-mediated apoptosis are β-catenin and p53 signalling which shall be discussed in greater detail in Chapter 5 and 6 respectively.

It is clear that NSAIDs affect multiple signalling pathways and it is possible that their potent anti-tumour activity results from multiple different effects on a variety of signalling pathways and genes. It is therefore important to unravel the primary chemopreventive effects from secondary phenomena.

1.2.3.3 Evidence supporting COX-independent anti-tumour mechanisms

Colorectal cancer cell lines that do not express COX are as susceptible to the growth inhibitory effects of NSAIDs as those which do have COX activity (Hanif *et al.*, 1996; Elder *et al.*, 1997). Similarly, NSAIDs lacking COX-2 inhibitory activity are able to inhibit colorectal cancer cell line growth (Piazza *et al.*, 1995; Piazza *et al.*, 1997b) . The concentration required to inhibit growth is greater than that required to inhibit COX activity (Hanif *et al.*, 1996; Charalambous *et al.*, 1998). Furthermore, formation of polyps was not completely abrogated in the COX-2 null mice (Oshima *et al.*, 1996), adding to the accumulating evidence that COX-2 is not the only target of NSAIDs.

There are conflicting reports regarding the cellular localisation of COX-2 in humans and animal models. COX-2 has been shown to be expressed in malignant epithelial cells in humans (Eberhart *et al.*, 1994; Sano *et al.*, 1995). A recent study showed that COX-2 localised to superficial interstitial macrophages in 75% of sporadic human adenomas and was only located in the epithelium of 29% of adenomas (Chapple *et al.*, 2000). In mice adenomas COX-2 was expressed in epithelial cells (Williams *et al.*, 1996) and also in the stromal rather than in the neoplastic epithelial cells (Oshima *et al.*, 1996; Shattuck-Brandt *et al.*, 2000). Non-neoplastic epithelium near the tumour also expresses COX-2 but that

distant from it does not, suggesting a paracrine signalling effect between stromal and epithelial cells (Hull *et al.*, 1999; Chapple *et al.*, 2000). The observation that, despite differing localisation of COX-2 in mice and humans, NSAIDs are anti-neoplastic in both species suggests that there may be alternative or additional mechanisms of action.

The transcription factor NFκB has been suggested as a target for the anti-inflammatory effects of NSAIDs (Kopp and Ghosh, 1994). Constitutively high NFκB activity has been observed in colorectal cancer suggesting that aberrant NFκB signalling may contribute to colon carcinogenesis (Rayet and Gelinas, 1999). Furthermore, several growth regulatory genes that are known to be deranged in colorectal cancer such as *c-myc, p53*, and *COX-2*, are regulated by NFκB. Hence, the NFκB signalling pathway is a strong potential target for the anti-tumour effects of NSAIDs in the colon.

1.3 Transcription factor nuclear factor κB (NFκB)

The NFκB/Rel proteins are members of a family of ubiquitous transcription factors that exist in all human cell types. NFκB was originally identified as a B-cell nuclear factor which was able to bind to an intronic enhancer of the immunoglobulin κ-light chain gene and thus named accordingly (Sen and Baltimore, 1986). Group 1 proteins NFκB1 (p50/p105) and NFκB2 (p52/p100) are produced following proteolysis of precursor proteins p105 and p100 respectively, and group 2 proteins include Rel A (synonomous with p65), RelB and c-Rel which all have transactivating domains. All five proteins share a common N-terminal 300 amino acid motif called the Rel homology domain (RHD) which is responsible for nuclear localisation, DNA binding, dimerisation and interacting

with IκB, the inhibitor of NFκB (Siebenlist *et al.*, 1994). The IκB family members (IκBα, IκBβ, IκBδ, IκBε, IκBγ, Bcl3) are characterised by the presence of ankyrin repeat domains (ARDs) in their C-termini, which are responsible for NFκB-IκB inhibitory interaction, of which IκBα is most studied (Baeuerle and Baltimore, 1988; Baldwin, Jr., 1996). The NFκB/Rel proteins can form various homodimers and heterodimers and the complex composition and cell type result in transcriptional specificity of target gene regulation. The most abundant form of NFκB is a heterodimer composed of a p65 (Rel A) and a p50 subunit (NFκB1) and further reference to NFκB will refer to this specific heterodimer unless otherwise stated. A schematic diagram of the canonical NFκB pathway is represented in Figure 1.5.

In resting cells, NFκB is sequestered pre-formed in the cytoplasm bound to its inhibitory protein, IκBα, and thus can be rapidly activated. IκBα preferentially recognises the NFκB (p50/p65) heterodimer and retains it in the cytoplasm, as a result of the direct interaction between the nuclear localisation sequence and DNA binding region of NFκB with IκBα (Malek *et al.*, 1998). In response to numerous different extracellular stimuli, a signal cascade is initiated culminating in activation of an enzyme complex which phoshorylates IκBα at two critical N-terminal serine residues at position 32 and 36 (Traenckner *et al.*, 1995). This multi-subunit enzyme complex has been identified as the IκB kinase (IKK) complex and is composed of two serine/threonine kinases, IKKα and IKKβ and additional proteins (Zandi *et al.*, 1997). Once IκBα has been phosphorylated, it undergoes polyubiquitination on lysines 21 and 22 by an E3 ubiquitin ligase, and is then targeted by the ubiquitin-26S proteosome for degradation (Traenckner and Baeuerle, 1995; Baldi *et*

al., 1996). IκBα degradation unmasks the nuclear localisation signal resulting in nuclear translocation of NFκB, where it binds to regulatory elements in promoters of target genes.

The NFκB transcription factor regulates several hundred target genes involved in cell proliferation and death, inflammation and the immune response (Pahl, 1999). NFκB plays a central role in regulating apoptotic pathways (Barkett and Gilmore, 1999) and dysfunction of NFκB signalling is implicated in the development of neoplasia (Elder and Paraskeva, 1998; Hawkey, 1999; Rayet and Gelinas, 1999). Furthermore, several NFκB driven genes are dysregulated in colorectal cancer supporting the notion that NFκB may be a potential NSAID target.

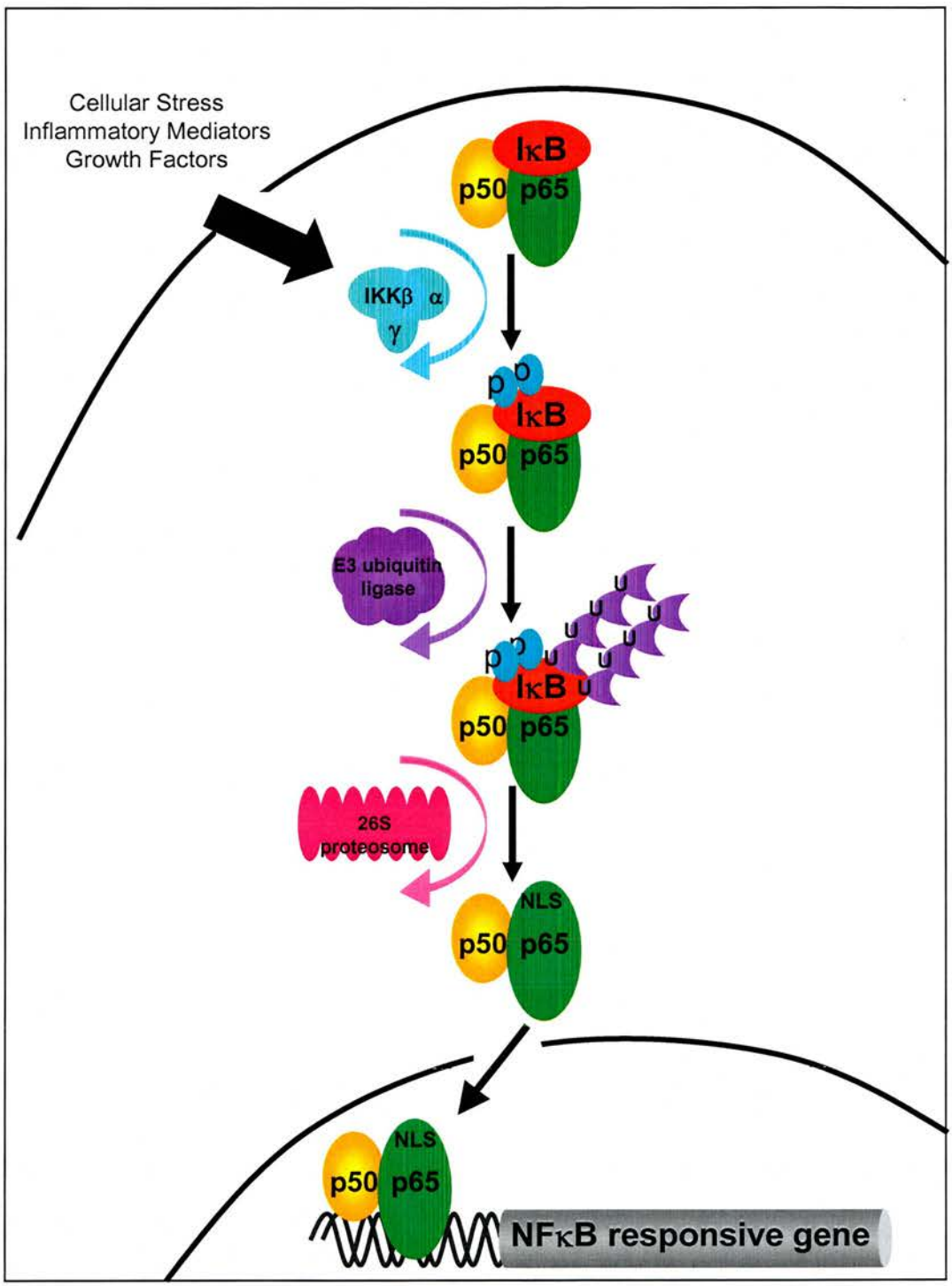

Figure 1.5 Schematic diagram of the NFκB signalling pathway.

NFκB is bound to an inhibitor protein IκBα in cytoplasm. Stimuli activate the IKK complex leading to IκBα phosphorylation at serines 32 and 36 and ubiquitination at lysines 21 and 22 by an E3 ubiquitin ligase, targeting IκBα for degradation by the 26S proteosome. IκBα degradation unmasks the nuclear localising signal (NLS) on p65 permitting NFκB nuclear translocation.

1.4 Research aims and strategy of research

1.4.1 Hypothesis

Aspirin exerts a clinically relevant anti-tumour effect via modulation of NFκB signalling.

1.4.2 Research questions

1. Is NFκB modulation responsible for aspirin-induced cell death in colorectal cancer?

2. Is the aspirin-induced NFκB molecular response specific to colorectal cancer?

3. Is there a role for COX-2 and β-catenin as potential molecular determinants of the aspirin-induced NFκB response?

4. Does p53 mutation and DNA mismatch repair status influence sensitivity to aspirin?

5. Are the effects observed *in vitro* reproducible in normal colonic mucosa and established tumours in patients?

6. Are there mutations present in the *Rel A* (p65) and *IkBa* genes in colorectal cancer?

1.4.3 Strategy of research

1.4.3.1 Cell culture studies

A panel of colorectal cancer cell lines will be treated with doses of aspirin relevant to chemoprevention. Following aspirin treatment, IκBα expression will be examined using Western blotting. Immunofluorescence will be used to study NFκB activity following

aspirin treatment. Apoptosis will be determined by immunocytochemistry and Annexin V binding. IκBα degradation and NFκB activation will be correlated with the level of apoptosis. Cell lines from other tumours will also be treated with aspirin to determine the specificity of the NFκB response for colorectal cancer. Basal levels of potential molecular markers for response such as COX-2, β-catenin, and p53 will be studied. To determine whether mismatch repair status alters the aspirin response, the colorectal cancer cell lines HCT-116 (mismatch repair deficient) and HCT-116^{+ch3} (mismatch repair proficient) will be compared.

1.4.3.2 Clinical studies

It is essential to confirm that the mechanism involving the NFκB signalling pathway observed in colorectal cancer cell lines represents the *in vivo* response to aspirin. The effects of NSAIDs will be studied in rectal cancer and genetically predisposed patients recruited to take a 7-day course of aspirin 300/600mg once daily (OD) or 600mg four times daily (QDS). The higher (analgesic) dose is designed to maximise generation of a biological effect, while the lower dose will allow determination of clinical relevance of the effect for future chemoprevention. To investigate the role of COX-2, the effects of a 7-day course of 25mg rofecoxib (a selective COX-2 inhibitor) on NFκB signalling will be studied in rectal cancer and genetically predisposed patients. All clinical studies have full ethical approval.

After informed consent, biopsies will be taken from rectal tumour and normal mucosa before and after treatment with the respective NSAID. Immunohistochemistry will be

used to study apoptosis in the biopsies after NSAID treatment. Cytoplasmic and nuclear protein extracts will be made using standard procedures. IκBα and p65 proteins will be examined in the cytoplasmic fraction using Western blotting. Electrophoretic mobility shift assays (EMSAs) will be used to study NFκB nuclear translocation. IκBα degradation and NFκB activation will be correlated with the level of apoptosis.

1.4.3.3 Mutation screening

The NFκB pathway plays a pivotal role in death signalling, and altered regulation of NFκB has been observed in colorectal cancers. It is hypothesised that deranged NFκB signalling in colorectal cancer may be due to mutations in key components of the NFκB pathway. Furthermore, mutations in the pathway may account for the variability in NFκB response observed in colorectal cancers and any cell-type specificity of the NFκB response. The principal objectives are to determine whether there are any mutations in *Rel A* and *IkBa* genes in colorectal cancer. Mutation analysis of *Rel A* and *IkBa* genes will be performed in 92 DNA samples. DNA will be extracted using standard protocols from colorectal cancer cell lines, colorectal tumours, aspirin-treated colorectal cancer patients, and genetically predisposed individuals with HNPCC and FAP. These biological resources are present within the group and sample collection will not be required.

Chapter 2

Materials and Methods

2.1 Biological Resources

2.1.1 Cell lines

The colorectal cancer cell lines used were HRT-18, SW480, HT-29, DLD-1, LoVo and HCT-116 and the sub-lines HCT-116^{+ch3} and HCT-116 $^{p53-/-}$. The HCT-116^{+ch3} sub-line was a gift from Professor CR Boland, in which *hMLH1* expression has been restored by chromosome 3 transfer, and these cells are DNA MMR proficient (Koi *et al.*, 1994). The HCT-116 $^{p53-/-}$ sub-line was created by targeted homologous recombination (Bunz *et al.*, 1999) and was a gift from Professor B Vogelstein. The breast cancer cell lines were T47D, MCF-7, MDA-MB-231; ovarian cancer line was A2780 and endometrial cancer line was HEC-1-A. The cell lines used are summarised in Table 2.1. All cell lines, excluding the HCT-116 sub-lines, are available from the American and European Type Culture Collections.

2.1.1.1 Generation of stable IκBα $^{S32/36}$ expressing cell lines

These cell lines were generated prior to my research period but are integral to establishing the mechanism of action of NSAIDs, and therefore a brief description is included here. The IκB $^{s32/36}$ plasmid was a gift from Professor R Hay and contains IκBα mutated at

serines 32 and 36, which are critical for phosphorylation and ensuing degradation, cloned into pcDNA3 with a C-terminal pk-tag (Roff *et al.*, 1996). The HRT-18 cell line was transfected with this plasmid and was grown under geneticin (Gibco BRL) selection. Stable transfectants were screened by immunoblotting, using the anti-pk-tag antibody. Expressing clones were grown in the absence of geneticin during aspirin treatment.

Table 2.1 Cell line characteristics

Cell line	Cancer Type	C0X-2	APC	β-catenin	p53	MMR
HRT-18	colon	unknown	mutant	wild-type	mutant	hMSH6
SW480	colon	negative	mutant	wild-type	mutant	proficient
HT-29	colon	positive	mutant	wild-type	mutant	proficient
DLD-1	colon	negative	mutant	wild-type	mutant	hMSH6
LoVo	colon	positive	mutant	wild-type	wild-type	hMSH2
HCT-116	colon	negative	wild-type	mutant	wild-type	hMLH1
HCT-116^{+ch3}	colon	negative	wild-type	mutant	wild-type	proficient
HCT-116$^{p53-/-}$	colon	negative	wild-type	mutant	null	hMLH1
MCF-7	breast	negative	wild-type	wild-type	wild-type	proficient
MDA-MB231	breast	positive	wild-type	wild-type	wild-type	unknown
T47D	breast	unknown	wild-type	wild-type	wild-type	proficient
A2780	ovarian	unknown	wild-type	wild-type	wild-type	proficient
HEC-1-A	endometrial	unknown	unknown	unknown	unknown	hMSH6 /hPMS2

2.1.2 Animal experiments

The technical staff in the animal facility were responsible establishing the xenografts and for maintaining the animals. Xenografts were established by subcutaneous implantation of a 0.1cm^3 piece of third to sixth generation source HT-29 tumours in both flanks of female Nu/Nu mice. The mice were euthanased 3-4 weeks later once the flank tumours

were approximately 50-100cm^3 in size and the tumours and other organs including small and large intestine, lung, liver, breast, spleen and pancreas were harvested. The organs were maintained *ex vivo* as short-term explants and treated with 10 mM aspirin. After washing in PBS, the biopsy tissue was finely diced and incubated in DMEM medium containing aspirin or carrier control for 5 hours. Following aspirin treatment, the biopsy fragments were harvested, washed in PBS, and homogensied in lysis buffer to permit cytoplasmic protein extraction as described in Section 2.6.2.1. Immunoblotting was as per the cell line protocol for cytoplasmic IκBα in Section 2.7. All animal experiments were performed in accordance with UKCCCR guidelines (Workman P, 1998).

2.1.3 Patients

There were three patient study groups: patients with established rectal cancers and patients who are genetically predisposed to developing colorectal cancer, such as those with FAP and HNPCC (Table 2.2). The inclusion criteria for the rectal cancer group were any patient over the age of 18 with a rectal cancer accessible using a rigid sigmoidoscope. The inclusion criteria for the genetically predisposed group were any patients over the age of 18 with known FAP or HNPCC. The exclusion criteria for all 3 study groups were current/recent ingestion of NSAIDs or anti-coagulant medication, pregnancy, known peptic ulcer disease or a tendency to indigestion / heartburn, allergy to NSAIDs, asthma, bleeding diastheses, previous stroke and those with a history of significant rectal bleeding. The treatment arms comprised either the COX-2 selective inhibitor rofecoxib or aspirin (300-600mg OD or 600mg QDS). All studies had local ethical and management approval (LREC numbers 99/5/8, 99/5/21, 99/5/50 and CTA Number 17844/0001/001).

After informed consent, biopsies were taken from normal rectal mucosa and rectal tumour, if present, before and after 7 days of respective NSAID treatment. Part of the biopsy specimen was fixed in formalin for paraffin embedding and sectioning for H&E staining, and part snap-frozen in liquid nitrogen for protein analysis. Cytoplasmic and nuclear proteins were extracted as described in Section 2.6.2.

Table 2.2 Clinical studies

Study	LREC no	Cases	NSAID type & dose	Patients recruited
Molecular studies of the anti-tumour effect of NSAIDs in colorectal cancer: implications for cancer therapy and prevention	1702/99/5/8	rectal tumours	Aspirin 300mg OD initially, changed to 600 mg OD	2 at 300 mg OD 4 at 600 mg OD
			Aspirin 600 mg QDS	6
Molecular studies of the anti-tumour effect of NSAIDs in colorectal cancer: implications for prevention in genetically predisposed individuals	1702/99/5/21	MMR	Aspirin 300mg OD initially, changed to 600 mg OD	2 at 300mg OD
		FAP	Aspirin 300mg OD initially, changed to 600 mg OD	1
		MMR	Aspirin 600 mg QDS	6
		FAP	Aspirin 600 mg QDS	2
Molecular studies of the anti-tumour effect of NSAIDs in colorectal cancer: chemopreventive action of rofecoxib, a cox-2 selective inhibitor	1702/99/5/50	rectal tumours	Rofecoxib 25mg OD	6
		FAP	Rofecoxib 25mg OD	6
		MMR	Rofecoxib 25mg OD	1

2.2 Cell line culture

Cells were grown as monolayers (37° C in 5% CO_2) in RPMI (HRT-18, DLD-1 and A2780), DMEM (HT-29, T47D, MCF-7, MDA-MB-231, HEC-1-A), L-15 (SW480) and McCoy's 5A media (HCT-116, HCT-116^{+ch3}, HCT-116 $^{p53-/-}$) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (media from Gibco BRL, Paisley, UK). The HCT-116 $^{+ch3}$ cell line was grown under selection with 0.4mg/ml geneticin.

2.3 Aspirin treatment

Cells were plated (1 x 10^6 cells /50ml flask) and grown until 60-70% confluent, prior to treatment with aspirin or carrier control. Aspirin (Sigma, St Louis, USA) was prepared as a 0.5 M stock solution in distilled water (final pH 7.0). Growth medium was replaced with the respective low serum (0.5% FCS) medium, and cells were treated with aspirin at 1,3,5 and 10mM for 24 hours or with carrier as a control.

2.4 Cell viability assessment

Adherent cells were harvested and viable cell number determined by haemocytometric counts with nigrosin exclusion. IC_{50} values for the colorectal cancer cell lines were calculated using the XL*fit* 3™ software.

2.5 Apoptosis measurement

2.5.1 Annexin V

Cell surface phosphatidylserine is a marker for apoptosis and was detected via its interaction with annexin V using the Annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, Mass., USA). Briefly, media from the flask of adherent cells was transferred to a conical tube on ice to harvest any floating cells. Cells were then washed with 2 mls of PBS, which was also added to the tube to collect any cells dislodged during washing. Cells were incubated with 1ml of trypsin: versene (volume per volume) just until the cells detached, and then resuspended in the conical tube containing the media with the floating and washed cells. Cells were counted using a haemocytometer and resuspended in cold 1X binding buffer to approximately 1 x 10^6 cells /ml. Media

binding reagent (10μl) was added to 0.5 ml of the cell suspension, which was incubated with 1.25 μl of annexin V-FITC for 15 minutes at room temperature in the dark. Annexin V was then removed by centrifugation at 1000 x g for 5 mins and cells were resuspended in 0.5 ml of cold 1X binding buffer and placed on ice. The counting was done using a haemocytometer (two counting grids) in duplicate, and this was carried out immediately.

2.5.2 Acridine orange staining

Following treatment with aspirin, adherent cells were fixed with acetic acid: ethanol (9:1 volume/volume) and then stained with acridine orange stain at 0.5% final concentration immediately prior to analysis by fluorescent microscopy by Dr L Stark.

2.5.3 Apoptotic counts

Dr Angus McGregor, Consultant Pathologist, kindly performed the apoptotic counts. Apoptosis was quantified on haemotoxylin and eosin (H&E) stained sections using morphological criteria and counting only epithelial cells showing unequivocal apoptotic morphology (Howie *et al.*, 1994; McGregor *et al.*, 2003). Since apoptosis is infrequent in tumour sections (< 1%), increasing the number of cells counted decreases the variation in apoptotic counts. The "running mean" method may be used to determine the minimum number of cells to be counted for the results to be reliable. In this method, the number of apoptotic cells per 100 malignant cells is calculated for every 100 malignant cells, until the difference between the successive means becomes negligibly small (< 1% of the index). In this work on rectal cancer samples, an initial calculation of a running mean

(cumulative mean of repeated counts, every 50 cells) was done with a cut-off at 0.5% (ie >0.5% difference likely to be significant) counting 1100 cells.

2.6 Protein extraction protocols

Solutions

Phosphate buffered saline

M $NaH_2PO_4.H_2O$

M $NA_2HPO_4.7H_2O$

pH 7.4

Lysis buffer

50 mM sodium chloride (NaCl)

10mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 8.0)

500 mM sucrose

1 mM EDTA

0.5 mM spermidine

0.15 mM spermine

0.2% w/v Triton X-100

Hypertonic buffer

350 mM NaCl

10 mM HEPES (pH8.0)

25% w/v glycerol

mM EDTA

0.5 mM spermidine

0.15 mM spermine

2.6.1 Cell lines

2.6.1.1 Cytoplasmic and nuclear extracts

Cells were washed with PBS, centrifuged (1200 rpm, 10 minutes) and cell pellets resuspended in lysis buffer containing complete Protease Inhibitor Cocktail and 100 mM Pefabloc (Roche Diagnostics, Manheim, Germany). The cell suspension was centrifuged (6000 rpm, 15 minutes, 4° C) and the supernatant containing cytoplasmic proteins aliquoted. Protein content was measured by the method of Bradford (BioRad, Hercules, California, USA).

2.6.1.2 Whole cell extracts

Cells were washed with PBS, centrifuged (1200 rpm, 10 minutes) and cell pellets resuspended in lysis buffer with 1:1000 DTT, 1:100 Pefabloc and 0.5% NP40 for 5 minutes and then centrifuged (3000 rpm, 10 mins). Supernatant was used as whole cell extract and protein content was measured by the method of Bradford.

2.6.2 Human and mouse tissues

2.6.2.1 Cytoplasmic and nuclear extracts

The biopsies of normal mucosa, tumour or mouse organ were washed in PBS and then diced into 0.5cm^3 pieces, which were suspended in 3 volumes of lysis buffer. The tissue and lysis buffer mixture then underwent three cycles of freeze (5 mins)- thawing and homogenising with an eppedorf pestle. Debris was removed by centrifuging (13000rpm, 5min) and then cytoplasmic and nuclear fractions prepared from supernatant as described above in the cell line protocol (Section 2.6.1.1).

2.7 Western blotting

Cytoplasmic proteins (30 μg) were separated on a 10% SDS-PAGE gel, transferred to a polyvinylidine difluoride membrane (BioRad) and blocked in 4% non-fat dry milk solution with 0.3% Tween20 (Sigma). Membranes were probed with a sheep polyclonal IκBα antibody (a gift from Professor R Hay, University of St Andrews, UK), rabbit polyclonal p65 antibody (Santa Cruz, California, USA), mouse monoclonal COX-2 antibody (Cayman Chemicals, Michigan, USA), mouse polyclonal β-catenin antibody (Chemicon, Hampshire, UK) and mouse monoclonal p53 antibody (AbCam, Cambridge, UK). Monoclonal antibody to copper zinc SOD (The Binding Site, Birmingham, UK) was used as a control for protein loading. Antigen-antibody complexes were visualised with chemiluminescence (Amersham ECL Reagents, UK).

2.8 Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were carried out by incubating nuclear extracts from untreated cells (6μg) with binding reaction mix (1X binding buffer [50mM KCl, 20mM HEPES, 5% glycerol, 1mM EDTA, 1mM DTT], 1μg BSA, 1μg poly dI-dC, 25 fmol radioactively labeled oligo-DNA in a final volume of 20μl) for 30min prior to analysis on a 4% native polyacrylamide gel. Double stranded oligonucleotides for NFκB were obtained from Santa Cruz.

2.9 Immunocytochemistry

Cells grown to 60-70% confluence on glass coverslips were treated with carrier or 10mM aspirin for 24 hours (in respective 0.5% FCS medium). After treatment, cells were washed with PBS, fixed with acetone: methanol (v/v) (–20° C, 10 minutes) and blocked in 10% pre-immune donkey serum (Sigma) for 1 hour. Rabbit polyclonal antibody to NFκB p65 (Santa Cruz) was applied for 1 hour followed by incubation with FITC-conjugated donkey anti-rabbit IgG for 1 hour. The nuclei were stained with DAPI and the coverslips mounted with Vectashield (Vector Laboratories, Burlingame, California, USA).

2.10 Transfections and reporter assays

For transient transfection experiments, 50ml flasks of cells were grown to sub-confluency (60-70%) and then transfected with 6μg of luciferase reporter plasmid and 3μg of β-galactosidase control plasmid, using Lipofectamine as described by manufacturers instructions (Gibco BRL). Following transfection, cells were grown in low serum (0.5% FCS) medium then treated with aspirin (0-10mM) for 24hrs. Luciferase activity was measured in cell extracts using a luciferase reporter assay kit (Promega) and read using a luminometer. Transfection efficiency and cell viability were monitored by co-transfection with a CMV-β-galactosidase reporter plasmid and β-galactosidase activity was quantified with an assay kit (Promega), as per manufacturers' instructions. Relative luciferase activity was calculated as unit of luciferase activity per unit of β-galactosidase activity.

2.11 DNA extraction protocols

2.11.1 Cell lines

Isolation of genomic DNA from cell lines was carried out using a Nucleon II extraction kit (Scotlab Bioscience, Strathclyde) according to manufacturer's instructions in a class 2 containment hood.

2.11.2 Paraffin-embedded sections

Tumour was micro-dissected from paraffin- embedded sections using a sterile scalpel and DNA extracted using a QIAamp DNA minikit (QIAGEN Ltd, Crawley, UK) as per manufacturer's instructions in a class 2 containment hood.

2.11.3 DNA concentration

DNA concentration was measured using a UV spectrophotometer (Pharmecia). Samples were diluted and placed in quartz cuvettes and absorbancy was measured at 260nm and at 280nm. DNA concentration was calculated as follows:

DNA μg/ml= A^{260} x dilution factor x 50

An A^{260} / A^{280} ratio of 1.8 was taken as optimum purity of DNA. Measurements were done in duplicate and repeated if inconsistent. Stock DNA samples were then diluted to 100-200ng/μl.

2.11.4 DNA samples from patients

Colorectal tumour DNA samples were previously collected by Prof. M Dunlop and Dr S Farrington (MRC Human Genetics Unit, Edinburgh). All samples have appropriate ethical approvals. The DNA samples were diluted to approximately 100ng/μl for use.

2.12 Mutation analysis of *Rel A* and *IkBa* genes

2.12.1 PCR protocols and primers

Manipulation of oligonucleotides and all PCR techniques were performed in a biological class 2 cabinet. Dedicated pipettes were used for PCR to minimise contamination of PCR products. All primers were specifically designed using Primer3 software (Whitehead Institute, Cambridge, MA http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

The primer sequences and cycling conditions are described in Table 2.3 for *Rel A* and Table 2.4 for *IkBa*.

Table 2.3 *Rel A* primers

Primers	Sequence	Cycling Conditions
p65 exon 1	GCGAATGGCTCGTCTGTAGT CTCCACATAGGGGCCAGAG	being optimised
p65 exon 2	ACTGTTCCCCCTCATCTTCC TCCAAACCTGACTCCCAAAC	being optimised
p65 exon 3	TTTGGGAGTCAGGTTTGGAT TACTTCATAGCCCGCCTCCT	52, **59*** and 62° C
p65 exon 4	AAGGTCTGGGCTCTGTGAGA GTGAGGGAGATGCAGGAAAG	52, **59,** 61 and 62° C
p65 exon 5	TGCCCCCACTGATAGTACCT AACCCCCTTCCTCCTATTCA	**55,** 58,59, 61 and 62° C
p65 exon 6	CTCTCGTGGCTCAGGTCATC CTTCTCAGCTTCACCCCTTG	**55,** 58,59, 61 and 62° C
p65 exon 7	GACCATCGGAGGGTTAGACA GTCTTGGCCTCTCTCTCACG	**59** and 62° C
p65 exon 8&9	TGAGGAGAGCAAGTCCCACT GCTGAACCTGTCGTTCCAGT	being optimised
p65 exon 10A	GGGTGGATCTCTAGGGCTTT GCCTCTGACAGCGTTCCTT	**62**° C
p65 exon 10B	AGCCATGGTATCTGCTCTGG ATCAGCATGGGCTCAGTTGT	?**52** and 62° C
p65 exon 10C	CTTGGCAACAGCACAGACC AAGTGCTTTTGGAGGGCTTC	**55** and 62° C

*-bold indicates optimum temperature

Table 2.4 *IkBa* primers

Primers	Sequence	Cycling Conditions
IkBa promoter	GTCCTTGGGATCTCAGCAG GGACTGCTGTGGGCTCTG	being optimised
IkBa exon 1A	CAGAGCCCACAGCAGTCC ACTTACGAGTCCCCGTCCTC	being optimised
IkBa exon 1B	CGAGGAGTACGAGCAGATGG GGCCTAGAGGACGGGTCT	being optimised
IkBa exon 2	AATAACCTCGCGGAAAACAC TACGTCCCAGGGTCAGAGAG	being optimised
IkBa exon 3	GGTTTGGTCCATGGCTTACT GCTCTTGCCTGGACTCCTTA	**58** and 62° C
IkBa exon 4	GGTGAAAGGAGTGAGGGTTG TAAGCACGAGGAGCCTGACT	55,**59**, 60,61& 62° C
IkBa exon 5	CAAATGCAGCCATAAGCATC AAGGGAATGGCACCTCATTA	55,**59**, 60,61& 62° C
IkBa exon 6	CTCCGGAAGCTTAACGTGTC GGCAGTGTGCAGTGTGGATA	55,**59**, 60,61& 62° C

PCR reactions were performed in a final volume of 50µl using AmpliTaq (Roche). Reaction mix per sample:

PCR Buffer II	10x	5µl	1x
$MgCl_2$	25 mM	5µl	2.5 mM
dNTP mix	2 mM	5µl	0.2 mM
AmpliTaq DNA polymerase	5U/µl	0.25µl	0.025 U
Forward primer	200ng/µl	0.5µl	100 ng
Reverse primer	200ng/µl	0.5µl	100 ng
DNA template		5µl	
Sterile H_2O (to 50µl)		28.75µl	

2.12.2 Agarose gel electrophoresis

Solutions

10x Tris-Acetate EDTA (TAE)	Loading buffer
2M Tris	100mM Na_2EDTA (pH 8.0)
5.7% w/v glacial acetic acid	0.25% w/v bromophenol blue
50mM Na_2EDTA (pH 8.0)	30% w/v sucrose

Agarose gels were prepared using electrophoresis grade agarose (Flowgen) and 1x TAE electrophoresis buffer. Low percentage gels (ie 1.5%) and higher percentage gels (ie 3%) were used to separate large and smaller DNA fragments respectively. 5-10 µl of PCR products were loaded on gels with 2µl of loading buffer and a 1kb size ladder (Gibco). The DNA was separated at 45V until the dye front was about 1 cm from the end of the gel. DNA was visualised by ethidium bromide (Biorad) staining followed by UV illumination using a Herolab transilluminator (Herolab, Wiesloch). Gel images were captured using a Herolab camera and Easywin 32 version 2 software.

2.12.3 PCR product purification

PCR products were cleaned for sequencing analysis using exonuclease 1 and shrimp alkaline phosphatase (USB, Cleveland, USA).

Reaction mix per sample:

Shrimp alkaline phosphatase	2µl
Exonuclease 1	1µl
PCR product	5µl

Samples were then transferred to an equilibrated thermal cycler (Hybaid) and run using the following cycling conditions:

37°C 15 minutes 1 cycle

80°C 15 minutes

4°C

2.12.4 DNA sequencing

Sequencing of purified PCR products was performed in 10µl reactions using ABI PRISM Ready Big Dye Terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Cheshire, UK). Reaction mix per sample:

Purified DNA template	2µl
Forward primer	1µl
Big dye	2µl
dH_2O	5µl

Samples were then transferred to a PCR system thermal cycler (Hybaid) and run using the following cycling conditions for 25 cycles:

96°C 30 seconds

50°C 15 seconds

60°C 4 minutes

4°C

Sequencing reactions were performed in either individual Eppendorf tubes or in 96-well plates and stored at 4°C prior to precipitation.

2.12.5 DNA precipitation

Precipitation of sequenced DNA was performed by adding 55µl 95% ethanol and 2 µl 3M NaOAC (pH 4) to 10µl of the sequencing reaction and incubating at room temperature for 30 minutes. After incubation, the 96 well plates were spun in a Sorvall RT6000 centrifuge at 2000 rpm for 30 minutes. The supernatant was removed by inverting the plate and then pulse spinning inverted plates on tissue paper at 800 rpm. 150µl of 70% ethanol was added to each sample and the plate inverted immediately. The pellets were further dried by a pulse spin of inverted plates on tissue paper at 800 rpm and stored at -20°C.

2.12.6 Gel electrophoresis of sequenced DNA

Gel electrophoresis of sequenced DNA was carried out by the Technical Services department at the MRC Human Genetics Unit, Edinburgh. The precipitated DNA pellets were resuspended in 2-4µl of loading dye (PE Applied Biosystems) and heated at 90°C for 2 minutes. The plates were incubated on ice until loading and were analysed on the ABI 3700 DNA Analyzer as per manufacturer's instructions.

2.12.7 Analysis of sequence data

Sequence data was imported into the Sequencher program version 3.0.1 (Gene Codes Corp., Michigan) to allow alignment of multiple sequences of the same fragment. The relevant published sequence was compared to sequenced fragments. The Sequencher program highlighted mismatches in individual base-pairs and the chromatograms for these were then examined.

2.13 Statistical methods

Statistical advice was provided by Dr Peter Teague (MRC, Human Genetics Unit, Edinburgh) who confirmed that the statistical test being employed was the most appropriate test to address any given null hypothesis. Wilcoxon signed rank test and Student's paired t-test (Microsoft Excel and Graphpad Prism Software) were used to test for differences between the distributions of two data sets. Rank and Pearson correlation coefficients (Microsoft Excel and Graphpad Prism Software) were calculated to describe the strength of an association between two variables.

Chapter 3

Molecular effects of aspirin on NFκB signalling in colorectal cancer

3.1 Introduction

Epidemiological data suggest that aspirin and other NSAIDs reduce the incidence and mortality from colorectal cancer (Janne and Mayer, 2000). The molecular mechanism of action responsible for the anti-tumour effects in colorectal cancer has not been fully defined. There are several potential targets and mechanisms of anti-tumour activity as discussed in Section 1.2.3. There is substantial rationale for the study of NFκB as an important mechanism of aspirin-mediated apoptosis. Constitutive NFκB activity has been observed in colorectal cancer, indicating that deranged NFκB signalling may promote carcinogenesis (Rayet and Gelinas, 1999). NFκB regulates many genes involved in cell proliferation and apoptosis, including genes known to be dysregulated in colorectal cancer such as *COX-2, c-myc* and *cyclin D-1*. There is also evidence that NSAIDs modulate the NFκB signalling pathway to exert their anti-inflammatory effects. Hence, the NFκB signalling pathway is a strong potential target for the anti-tumour effects of NSAIDs in the colon. In resting cells, NFκB is retained in an inactive cytoplasmic complex by IκBα, an inhibitory protein. The NFκB-IκBα complex can shuttle between the nucleus and cytoplasm but nuclear export is more efficient, and hence the complex is

mainly cytoplasmic. Following stimulation, an enzyme cascade is triggered activating the IKK enzyme complex, which phosphorylates IκBα at serine residues 32 and 36. Once phosphorylated, IκBα is ubiquitinated at lysines 21 and 22, and this polyubiquitation targets the protein for degradation by the 26S proteosome. Following IκBα degradation, NFκB is free to translocate to the nucleus where it regulates target gene transcription.

Aspirin and its active metabolite sodium salicylate have been shown to inhibit the activation of NFκB in T and pre-B lymphocytes, by inhibiting degradation of lipopolysaccharide-induced IκB, and thereby preventing NFκB nuclear translocation (Kopp and Ghosh, 1994). Glutamate-mediated induction of NFκB is inhibited by aspirin and sodium salicylate, and hence protects against glutamate-induced neurotoxicity in rat neuronal cells (Grilli *et al.*, 1996). It has also been reported that aspirin inhibits tumour necrosis factor α (TNFα)-induced NFκB activity in human endothelial cells (Weber *et al.*, 1995). The common feature in these studies is that NSAIDs have been shown to inhibit cytokine-induced NFκB activation. The mechanism of inhibition of NFκB activity has been attributed to competitive inhibition of ATP-binding to the IκB kinase (IKKß) by aspirin and sodium salicylate in TNFα pre-treated COS and Jurkat cells (Yin *et al.*, 1998). However, high concentrations of NSAIDs have been shown to inhibit other cellular kinases, and so the specificity of this effect for NFκB is unclear (Frantz and O'Neill, 1995). Subsequently, the NSAID sulindac was shown to reduce TNFα-mediated increases in IKKβ activity in HCT-15 and HT-29 colorectal cancer cells (Yamamoto *et al.*, 1999b). Hence, inhibition of IKKβ and subsequent inhibition of NFκB signalling have been reported as being responsible for the anti-tumour effects of NSAIDs in colorectal cancer.

NFκB has been shown to act as an inducer or inhibitor of apoptosis, depending on cell-type and the nature of stimulus (Fan *et al.*, 2002). NFκB is constitutively active in numerous cancer types and appears to confer a degree of resistance to apoptosis, potentially by upregulating anti-apoptotic genes. Both constitutive NFκB activity and NFκB activation in response to chemotherapy may be responsible for resistance to chemotherapeutic agents (Wang *et al.*, 1999a). Therefore, inhibition of NFκB activity may increase susceptibility to apoptosis. However, in the studies showing NSAID-mediated inhibition of IKKβ, cells were pre-treated with NSAIDs for a short period prior to a burst of TNFα. Such experimental conditions are not representative of the *in vivo* environment in which colonic epithelium is continuously exposed to NSAIDs. Therefore, the relevance of these studies to the chemopreventive effects of NSAIDs is unclear.

The aim of this work was to establish whether, in the absence of TNFα or other NFκB-inducing agents, NSAIDs mediate an anti-tumour effect by targeting the NFκB pathway. Therefore, these experiments were performed *without* the addition of cytokines, to identify the underlying role of NFκB in NSAID-mediated apoptosis. This research was being performed by Dr Stark in the host laboratory when I joined. My contribution to this body of work was the studies at low aspirin concentrations (0, 0.5, 1, 2 mM), which included assessment of cell viability and apoptosis, IκBα degradation immunoblots and NFκB nuclear translocation EMSAs following aspirin treatment. It was important to investigate these effects at concentrations of aspirin relevant to those found in serum of aspirin-treated patients. In addition, I harvested the normal and tumour tissue samples from patients with colorectal cancer and performed the explant studies using aspirin.

3.2 Overview of Methods

3.2.1 Cell line studies

SW480, HRT-18, and HCT-116 colorectal cancer cell lines were maintained and treated with aspirin for 24 hours, as described in Section 2.2 and 2.3. Colorectal cancer cells expressing IκBα mutated at serines 32 and 36 were generated as stable and transient transfectants as described Section 2.1.1.1. Following treatment with aspirin, cell viability and apoptosis were assessed by haematocytometric counting and annexin V assaying which are described in Sections 2.4 and 2.5.1 respectively. Morphological assessment of apoptosis was analysed by acridine orange staining (Section 2.5.2). Western blotting was used to determine the levels of cytoplasmic IκBα (Section 2.7) and NFκB nuclear translocation was studied using immunocytochemistry and EMSAs (Section 2.8 and 2.9).

3.2.2 Clinical studies

For the *ex-vivo in-vitro* studies, biopsies of normal mucosa and tumour were harvested from patients undergoing resectional surgery for colorectal cancer. After washing in PBS, the biopsy tissue was finely diced and incubated in RPMI medium containing aspirin or carrier control for 5 hours. Following aspirin treatment, the biopsy fragments were harvested, washed in PBS, and homogenised in lysis buffer to permit cytoplasmic protein extraction as described in Section 2.6.2.1. Immunoblotting was as per the cell line protocol for cytoplasmic IκBα in Section 2.7.

3.3 Results

3.3.1 Aspirin induces apoptosis in colorectal cancer cells

The effects of aspirin on colorectal cancer cell viability were studied using the SW480 cell line. Cells were treated with 1-10mM aspirin for 24 hours or 0.5-2mM aspirin for 48 hours. Following aspirin treatment, there was a concentration-dependent reduction in the number of viable cells (Figure 3.1A&B). Apoptosis was studied to establish whether the effects of aspirin were due to the induction of cell death, rather than a growth inhibitory effect. Indeed there was a concentration-dependent increase in the proportion of cells showing annexin V binding of phophatidylserine, a marker of apoptosis, following treatment with aspirin (Figure 3.1A&B). Acridine orange staining of aspirin-treated cells demonstrated morphological features of apoptosis including condensed cytoplasm, granular chromatin and irregular condensed nuclei (Figure 3.1C). These results confirm that aspirin induces apoptosis in colorectal cancer cells.

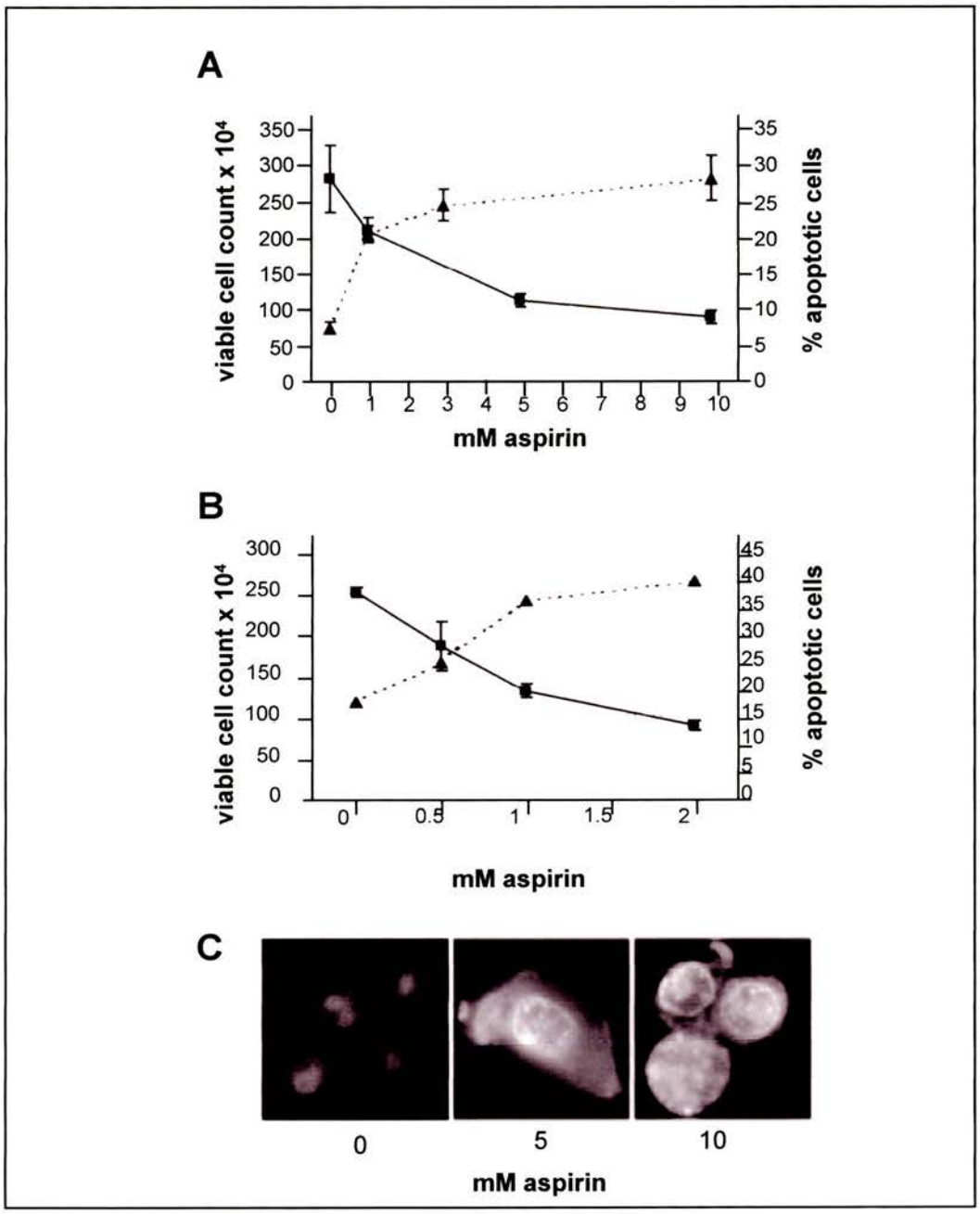

Figure 3.1 Aspirin induces apoptosis in colorectal cancer cells. SW480 cells were treated with 1-10mM aspirin for 24 hours (**A**) or 0.5-2mM aspirin for 48 hours (**B**). There was a concentration-dependent decrease in the viable cell count paralleled by a concentration-dependent increase in the proportion of cells binding annexin V (**A&B**). Aspirin-treated cells show morphological features of apoptosis after acridine orange staining (**C**).

3.3.2 Aspirin induces IκBα degradation and NFκB nuclear translocation in a concentration-dependent manner in colorectal cancer cells

The effect of aspirin on the NFκB pathway was studied to determine whether aspirin-induced apoptosis is associated with modulation of NFκB signalling. Since IκBα regulates NFκB by sequestering it in the cytoplasm, the effects of aspirin on IκBα were studied initially. Aspirin treatment for 24 or 48 hours induced degradation of cytoplasmic IκBα levels in a concentration-dependent manner in SW480 colorectal cancer cells (Figure 3.2 A). Normally, upon stimulation IκBα is phosphorylated at serines 32 and 36, and then ubiquitinated, which targets the protein for degradation by the 26S proteosome. Transfection experiments performed in SW480 cells using a dominant negative form of IκBα ($IκB^{S32/36}$-tag), which is resistant to phosphorylation at the critical serine residues and hence degradation, showed that aspirin treatment did not alter the levels of mutant IκBα protein (Figure 3.2B). This indicated that the phosphorylation sites are important for the effect of aspirin on IκBα. Furthermore, the aspirin-induced decrease in IκBα levels was abrogated by pre-incubation of SW480 cells with the MG132 proteosome inhibitor (Figure 3.2C). Taken together, these results show that aspirin induces phosphorylation and proteosome-mediated degradation of IκBα.

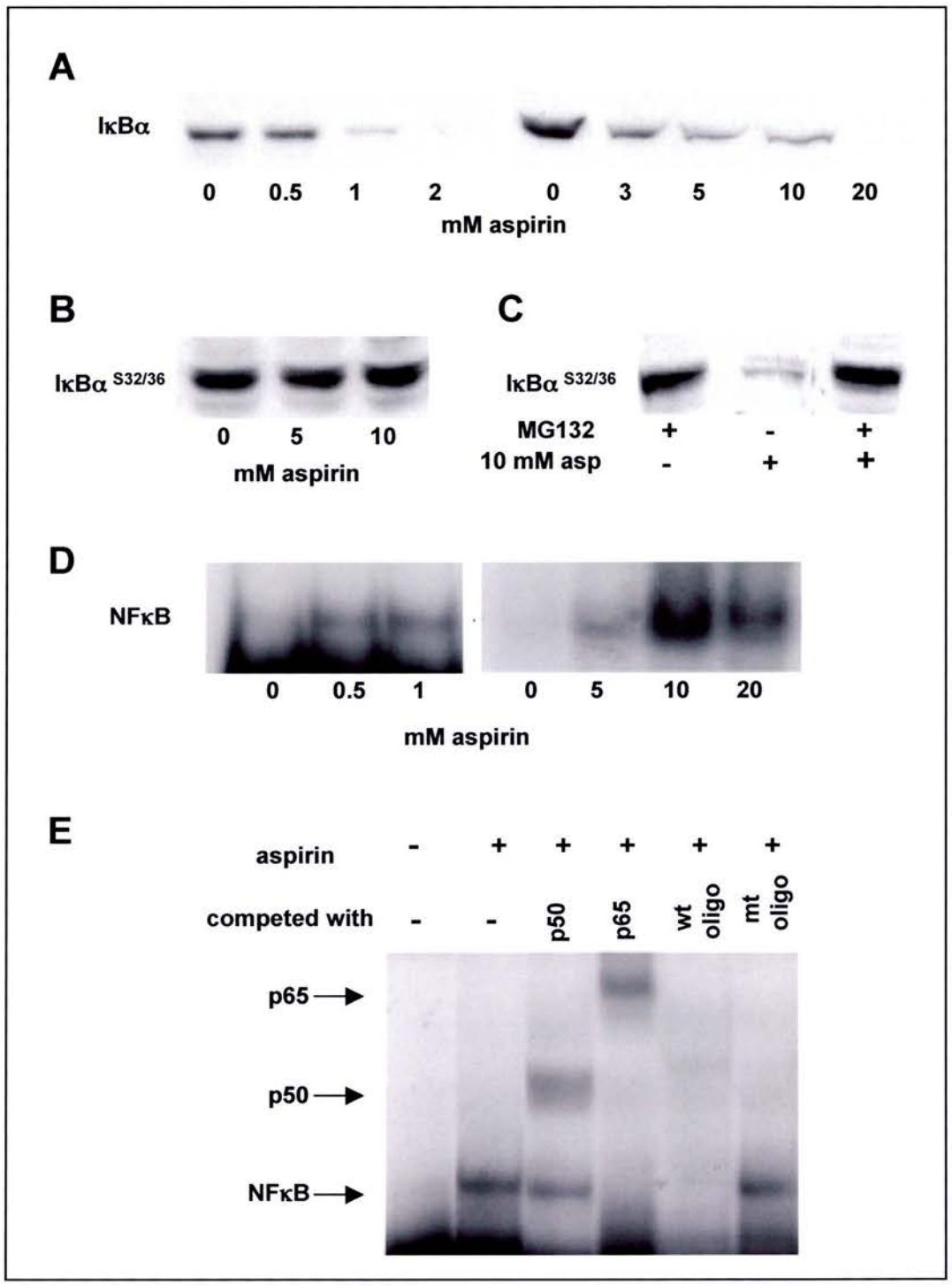

Figure 3.2 Aspirin induces IκBα degradation and NFκB nuclear translocation in colorectal cancer cells. Aspirin (24-48 hours) induced IκBα degradation in SW480 cells (**A**). Aspirin does not induce degradation of mutant IκBα, resistant to phosphorylation(IκB $\alpha^{S32/36}$)(**B**). Aspirin-induced decrease in IκBα is abrogated by pre-incubation with MG132 proteosome inhibitor (**C**). Aspirin induces NFκB nuclear translocation (**D**).Super-shift analysis shows the induced complex is p65 and p50 (**E**).

Dissociation of IκBα from NFκB normally permits translocation of NFκB from the cytoplasm to the nucleus. EMSAs were used to examine whether nuclear translocation of NFκB occurs following aspirin-induced IκBα degradation. Indeed, aspirin-induced IκBα degradation was associated with nuclear translocation of NFκB in a concentration-dependent manner (Figure 3.2D) and super-shift analysis revealed the induced complex to be composed of p65 and p50 sub-units (Figure 3.2E). These results show that aspirin induces IκBα degradation and nuclear translocation of NFκB in colorectal cancer cells.

3.3.3 Kinetics of aspirin effects on NFκB signalling and apoptosis

It is important to determine whether the observed effect of aspirin on IκBα and NFκB *results* in apoptosis, rather than being a consequence of it. The first piece of evidence comes from time-course experiments in which aspirin treatment induced degradation of IκBα after 2-5 hours, which persisted beyond 24 hours (Figure 3.3A). Increased nuclear NFκB DNA binding occurs 2 hours after treatment and persists for more than 16 hours (Figure 3.3B). Hence, both IκBα degradation and NFκB nuclear translocation occur prior to apoptosis, which increases between 8 and 16 hours following aspirin treatment (Figure 3.3C) indicating a causal relationship between aspirin-induced effects of NFκB signalling and programmed cell death.

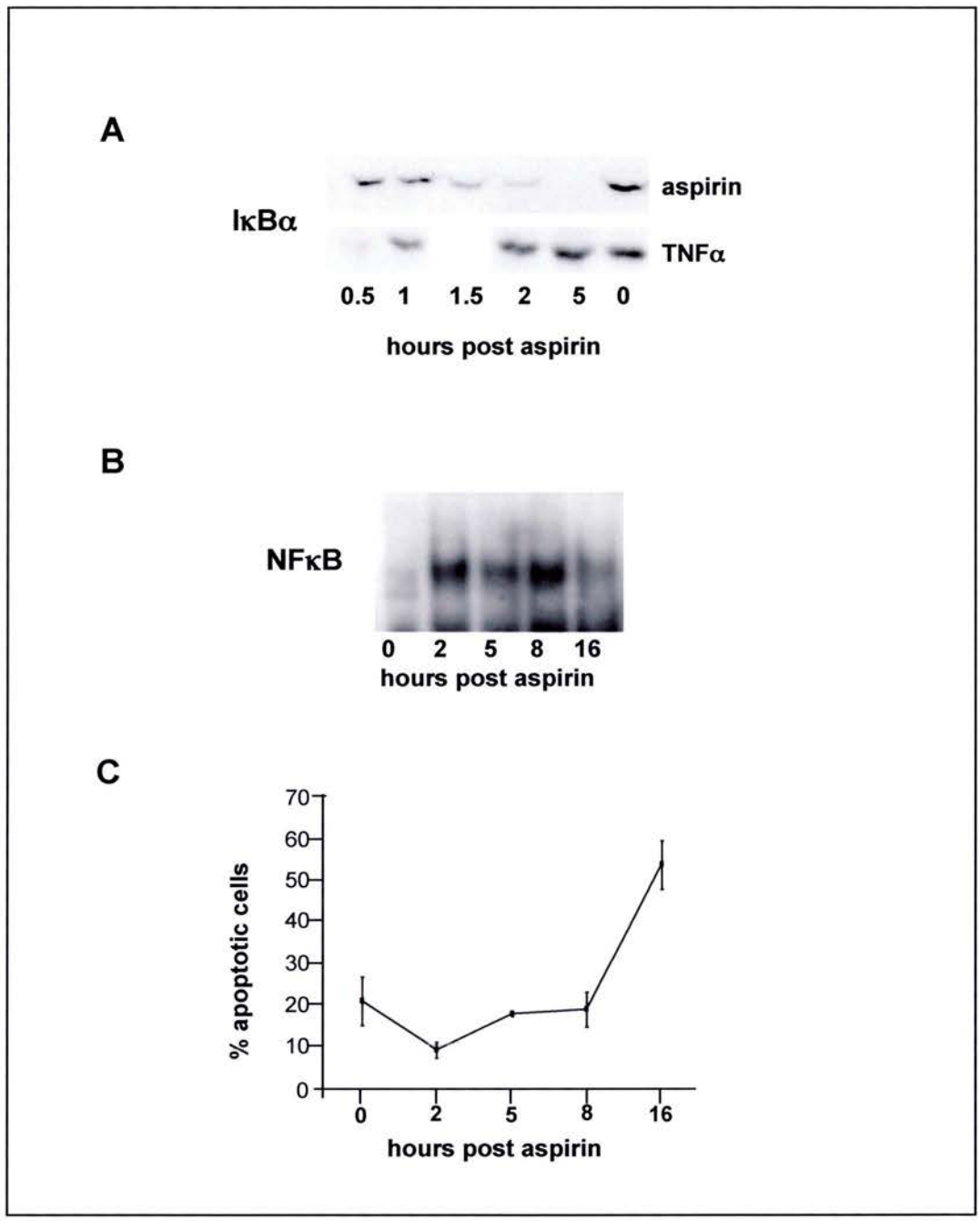

Figure 3.3 Kinetics of aspirin effects on NFκB signalling and apoptosis
Aspirin treatment induced IκBα degradation after 2-5 hours (**A**) and there is increased nuclear NFκB DNA binding at 2 hours which lasts for greater than 16 hours (**B**). Aspirin induced apoptosis occurs between 8 and 16 hours following treatment (**C**).

3.3.4 Aspirin-induced apoptotic response and NFκB nuclear translocation is abrogated by mutant IκBα

The next experiment was designed to establish whether there was a causal relationship between NFκB nuclear translocation and aspirin-mediated apoptosis. The HRT-18 colorectal cancer cell line was stably transfected to constitutively express the IκBα $^{S32/36}$ -tag construct in which IκBα is mutated at serines 32 and 36 with a C-terminal pk-tag. The parental HRT-18 cell line showed substantial NFκB nuclear translocation following aspirin treatment (Figure 3.4A). However, nuclear translocation of NFκB was inhibited in all clones expressing mutant IκBα compared to parental cells (Figure 3.4A). Next, the effects of aspirin on cell viability and apoptosis were examined. Interestingly, the HRT-18 clones expressing IκBα $^{S32/36}$ (h1, h28) grew in the presence of 1 mM aspirin compared with a decrease in viable cell count in parental HRT-18 (Figure 3.4B). Treatment with 5 mM aspirin also had significantly less effect ($p<0.05$, Student's t-test) on the viability of mutant IκBα expressing clones, compared to the parental colorectal cancer cells (Figure 3.4B). These results show that abrogation of nuclear translocation of NFκB protects colorectal cancer cells from aspirin-induced apoptosis. These data strongly support the concept that aspirin-induced apoptosis of colorectal cancer cells depends on nuclear translocation of NFκB, which is a consequence of phosphorylation and degradation of IκBα.

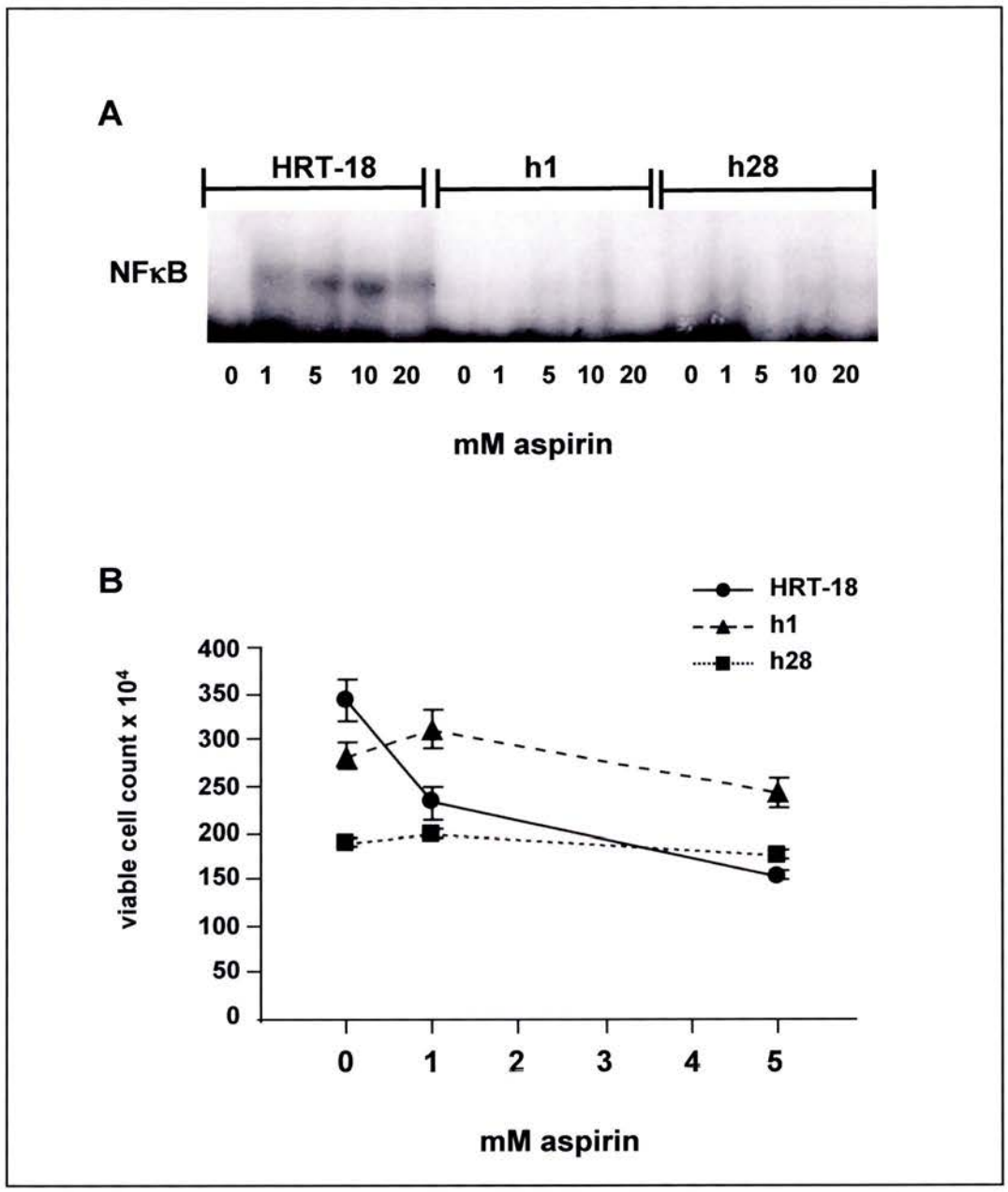

Figure 3.4 Aspirin-induced apoptosis and NFκB nuclear translocation is abrogated by mutant IκBα. HRT-18 cells stably transfected to constitutively express IκBα $^{S32/36}$ - tag construct (mutated at serines 32 & 36). EMSA shows that NFκB translocates to nucleus following aspirin treatment in parental HRT-18 cells but is inhibited in clones (h1 & h28) expressing mutant IκBα (**A**). There is a decrease in viable cell count in parental HRT-18 cells, but clones expressing IκBα $^{S32/36}$ grew in 1 mM aspirin, and 5 mM aspirin had significantly less effect ($p<0.05$) on cell viability (**B**).

3.3.5 Aspirin induces IκBα degradation in colorectal tumour samples treated with aspirin

Next, the potential clinical significance of the *in vitro* findings was investigated by treating normal mucosa and rectal tumour biopsy samples from patients, undergoing resection for rectal cancer, with aspirin. The biopsies were maintained *ex vivo* as short-term explants and treated with 10 and 20 mM aspirin. There was IκBα degradation in tumour and to a lesser extent normal mucosa following 5 hours of treatment (Figure 3.5). This experiment confirms that clinical tumour biopsy material shows the same IκBα response observed in cell line experiments, albeit in an artificial *in vitro* experimental environment. However, the effect of aspirin on NFκB signalling *in vivo* is expanded in Chapter 7.

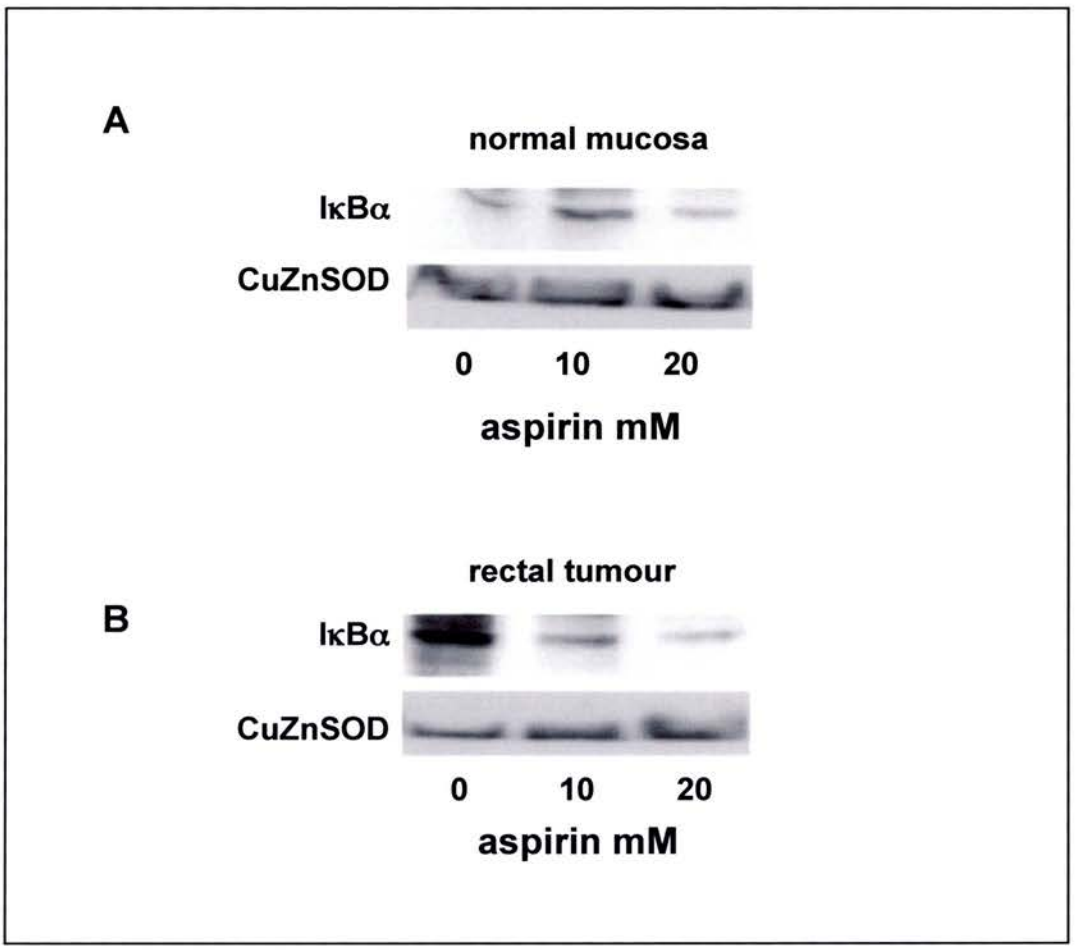

Figure 3.5 Aspirin induces IκBα degradation in colorectal tumour biopsies.
Normal mucosa and rectal tumour biopsies were maintained *ex vivo* as short-term explants and treated with 10 and 20 mM aspirin. Aspirin induces IκBα degradation in tumour (**B**) and to a lesser extent normal mucosa (**A**) following 5 hours treatment. The Western blots shown are representative of at least 3 independent experiments and Cu/Zn SOD was used as a control for protein loading.

3.4 Discussion

Despite substantial evidence that NSAIDs protect against colorectal cancer, the molecular basis of this anti-tumour activity has not been fully elucidated. The work presented here shows that aspirin has growth inhibitory effects in colorectal cancer cells due to the induction of programmed cell death. Aspirin-induced apoptosis is paralleled by degradation of IκBα and by translocation of NFκB to the nucleus in a concentration-dependent manner. Colorectal cancer cells expressing a super-repressor form of IκBα ($IκBα^{S32/36}$) do not undergo aspirin-induced NFκB nuclear translocation resulting in complete abrogation of apoptosis; confirming that IκBα degradation and NFκB nuclear translocation are required for aspirin-induced apoptosis. These data reveal a novel mechanism of action for the anti-neoplastic effects of NSAIDs against colorectal cancer.

Although the underlying mechanisms of anti-tumour activity are incompletely defined, several studies show that NSAIDs inhibit growth of colorectal cancer cells (Shiff *et al.*, 1995; Elder *et al.*, 1996; Ricchi *et al.*, 1997; Castano *et al.*, 1999). Potential mechanisms responsible for NSAID-induced growth inhibition are cell cycle arrest, induction of apoptosis and necrosis. The work presented in this chapter shows that aspirin induces apoptosis observed by morphological changes such as nuclear chromatin condensation and externalisation of phosphatidylserine. Nevertheless, the preceding molecular interactions that culminate in NSAID-induced cell death are unclear and require further investigation. The anti-inflammatory effects of NSAIDs are due to COX-inhibition and decreased prostaglandin synthesis (Vane, 1971), and it was thought that this mechanism may also underly the anti-tumour activity. However, mounting evidence from animal

models and cell culture systems, described in Section 1.2.3.3, has shown that it is likely that COX-2-independent mechanisms of action play an important role in NSAIDs anti-tumour activity.

Aspirin-induced apoptosis in colorectal cancer cells subsequent to activation of NFκB signalling, in terms of IκBα degradation and NFκB nuclear translocation, contrasts previous studies that show inhibition of NFκB following NSAIDs (Yin *et al.*, 1998; Yamamoto *et al.*, 1999b). These studies showed NSAID-mediated inhibition of IKKβ in cells that were pre-treated with NSAIDs for a short period prior to a burst of TNFα to activate NFκB signalling. However, given that colonic epithelium would be constantly exposed to NSAIDs, these conditions are not considered to be representative of the *in vivo* dynamics, and NSAIDs have been shown to inhibit colorectal cancer cell growth without additional cytokine stimulation. Furthermore, a recent study reported that the direct inhibitory effect of sodium salicylate upon IKKβ activity in a cell-free model such as kinase assays does not reflect the mechanism whereby sodium salicylate inhibits IKKβ activity in intact cells (Alpert and Vilcek, 2000). Therefore, although these previous studies provide some mechanistic insights relevant to NFκB signalling, the importance of these findings using TNFα stimulation to chemopreventive effects of NSAIDs is unclear.

The experiments were performed in low-serum conditions since components of high serum may activate the NFκB pathway. In addition, experiments examining the effects of cytokines on NFκB have been traditionally incubated in low concentration serum prior to treatment. However, serum withdrawal has been shown to promote apoptosis by NFκB

nuclear translocation depending on the cell context (Campbell *et al.*, 2004). Hence, it may be possible that the NFκB and apoptotic responses to aspirin may be either due to or being enhanced by the low serum conditions. Further work in the host laboratory has shown that there is no difference in NFκB and apoptotic responses to aspirin which is attributable to differing serum concentrations when colorectal cancer cells are treated in 0.5% and 10% FCS (Stark and Dunlop, 2005).

The higher concentrations of aspirin (5-10 mM) were used to maximise the likelihood of generating a biological effect, while the lower concentrations (0.5-3 mM) determined the clinical relevance. The lower aspirin concentrations used here are comparable to serum levels achievable in patients taking aspirin for chronic inflammatory conditions (1-3mM) (Pachman *et al.*, 1979). Although the higher concentrations are greater than serum levels, there is some evidence that local conditions may increase uptake and concentration of salicylates (Alpert and Vilcek, 2000). Salicylates are organic acids and therefore accumulate at mildly acidic environments such as sites of inflammation (Abramson and Weissmann, 1989; Weissmann, 1991). This may be due to salicylates being uncharged at low pH and hence readily crossing membranes, but then deprotonating and becoming trapped as anions within the more neutral environment found within cells (Brooks and Day, 1991). Hence, it is likely that the local concentration of salicylate at a site of inflammation may reach levels greater than serum concentrations.

Since aspirin is largely hydrolysed to salicylate on first pass metabolism, it may be debated that the effects of salicylate rather than aspirin should be investigated. Indeed, data from the host laboratory show that sodium salicylate has the same effects on NFκB

signalling as aspirin (Stark *et al.*, 2001a). Nonetheless, the effects of aspirin on cell viability, apoptosis and NFκB signalling are evident at the lowest concentration of aspirin (1 mM), which is an achievable serum level in patients and hence relevant to long-term chemopreventive use. Furthermore, the observation that the colorectal tumour tissue explants respond in a similar manner to aspirin-treated colorectal cancer cells demonstrates proof of principle, in terms of aspirin-mediated modulation of NFκB signalling *in vivo*. Hence, it is important to perform the experiments with aspirin, as this is the main agent that has been shown to be protective in the epidemiological studies, and any clinical studies would use aspirin since patients cannot be given salicylate.

In summary, these results show that aspirin induces a concentration and time-dependent reduction in cytoplasmic IκBα in colorectal cancer cells that is due to phosphorylation-dependent, proteosome-mediated degradation of the protein. The aspirin-induced IκBα degradation is associated with nuclear translocation of NFκB, as determined by electrophoretic mobility shift assays. Time-course and dose-response experiments aimed at defining the kinetics of the aspirin effect indicate that IκBα degradation and NFκB nuclear translocation preceded cell death, suggesting a causal relationship. Furthermore, inhibition of NFκB nuclear translocation in colorectal cancer cells generated to constitutively express a super-repressor IκBα ($I\kappa B\alpha^{S32/36}$) completely abrogates aspirin-induced apoptosis compared to their parental counterparts. These results identify a novel mechanism of aspirin-mediated apoptosis involving the NFκB pathway.

Chapter 4

Investigation of the cell specificity of the NFκB response to aspirin

4.1 Introduction

Combined case-control data, including over 30,000 colorectal cancer cases, indicate a 45% reduction in risk of developing colorectal cancer in subjects taking NSAIDs. Although there is evidence for a protective effect of NSAIDs against non-gastrointestinal cancers, the data are less convincing and the risk reduction much less. In breast cancer, reports show conflicting results and a meta-analysis revealed a risk reduction of only 13% in case-control studies (Khuder and Mutgi, 2001), considerably less than that in colorectal cancer. Similarly, in endometrial and ovarian cancer the available evidence suggests that NSAIDs confer little, if any, protection (Cramer *et al.*, 1998; Rosenberg *et al.*, 2000; Fairfield *et al.*, 2002; Meier *et al.*, 2002). Collectively, published data suggests that there is considerable heterogeneity of NSAID anti-tumour effects *between* cancer types. The particular protective effect against colorectal cancer suggests the possibility that aspirin might target distinct molecular pathways in colonic epithelial cells.

The previous work presented in Chapter 3 demonstrated that aspirin induces time- and dose-dependent signal-specific degradation of IκBα, nuclear translocation of NFκB and

apoptosis in colorectal cancer cells. Time-course experiments indicated that IκBα degradation and NFκB nuclear translocation preceded cell death indicating a causal relationship. This was confirmed in cells that were engineered to continuously express a dominant negative mutant IκBα ($IκBα^{S32/36}$), which showed inhibition of both aspirin-induced NFκB nuclear translocation and apoptosis compared to their parental counterparts (Stark *et al.*, 2001b). This work alluded to the notion of specificity since the NFκB response was not observed in the control cell lines, which were non-colonic in origin.

Here, the focus is on the important issue of the specificity of aspirin's protective effects, as observed in epidemiological studies, and the aim was to investigate whether cell-type specific effects on the NFκB signalling pathway reflect the differential protective effects of aspirin in different cancer types. Firstly, the generality of the NFκB response to aspirin in colorectal cancer was investigated by studying a panel of colorectal cancer cell lines with different genetic defects common in bowel malignancy. The key aim was to determine whether the lower protective effect observed for breast, ovarian and endometrial cancer in epidemiological reports might be paralleled by differing effects of aspirin on the NFκB signalling pathway. The specificity of the NFκB apoptotic response was investigated by comparing the effects of NSAIDS in colorectal cancer cell lines to a panel of cancer cell lines of non-colonic origin.

4.2 Overview of Methods

4.2.1 Generality of NFκB response in colorectal cancer

The generality of the NFκB response in colorectal cancer was examined in panel of 6 colorectal cancer cell lines: HRT-18, SW480, HT-29, DLD-1, LoVo and HCT-116. These cell lines have different genetic backgrounds with mutations in genes that are known to be involved in colorectal cancer development (Table 2.1).

All cell lines were maintained as described in Section 2.2 and grown until 60-70% confluent, prior to treatment with aspirin or carrier control at the same concentrations (1,3,5 and 10mM) as the aspirin treatment (Section 2.3). Following aspirin treatment for 24 hours, adherent cells were harvested and the number of viable cells determined (Section 2.4). Apoptosis was detected via the interaction of phosphotidylserine, which is externalised during apoptosis, and annexin V (Section 2.5.1). Protein extracts were made from cell lines after aspirin treatment as described in Section 2.6.1 and proteins electrophoresed as described in Section 2.7. The Western blots were then probed with antibody to IκBα to detect changes in cytoplasmic levels. Copper zinc SOD antibody was used as a loading control. Nuclear translocation of NFκB was detected by immunofluorescence using antibody to p65, the transcriptionally active subunit of NFκB as described in Section 2.9.

4.2.2 Specificity of NFκB response to colorectal cancer

To determine whether the effects of aspirin on cell viability and NFκB signalling were specific to colorectal cancer, the panel of colorectal cancer lines was compared to cancer cell lines of non-colonic origin. The non-colorectal cancer cell lines were breast cancer lines (T47D, MCF-7, MDA-MB-231); an ovarian cancer line (A2780) and an endometrial cancer line (HEC-1-A). These non-colorectal cancer cell lines were exposed to aspirin in an identical manner to the colorectal cancer panel and cell viability, apoptosis, IκBα degradation and NFκB nuclear translocation determined (as described above). Basal levels of IκBα and p65 proteins were examined in extracts from untreated cells from both panels and quantified using densitometry to study whether differences in specificity may be related to the constituents of the NFκB pathway. Copper zinc SOD antibody was used as a loading control.

To further investigate the specificity of the aspirin-induced NFκB response, HT-29 tumour xenografts and other organs including breast, lung, liver, pancreas, spleen, small and large intestine were harvested from mice and maintained *ex vivo* as short term explants. The explants were treated with aspirin or carrier control for 5 hours, following which cytoplasmic protein extracts were made as described in Section 2.6.2 and IκBα levels examined.

4.3 Results

4.3.1 Generality of NFκB response in colorectal cancer

4.3.1.1 Aspirin induces apoptosis in colorectal cancer cells

In triplicate dose-response experiments, cell lines were treated for 24 hours with aspirin at concentrations of 1,3, 5 and 10 mM and viable cell number determined by haemocytometric counts. There was a concentration-dependent decrease in viable cell number in each of the six colorectal cancer cell lines studied (Figure 4.1A). The IC_{50} values were calculated from the growth curves of the aspirin-treated colorectal cancer cell lines (Table 4.1). The mean IC_{50} value for the colorectal cancer cell lines was 2.38 mM and the greatest incremental reduction in viability in these cells was observed between 0 and 1mM concentrations, which is comparable to serum concentrations attainable in humans (Pachman *et al.*, 1979).

Table 4.1 IC_{50} values for colorectal cancer cell lines

Colorectal cancer cell line	IC_{50}
HRT-18	3.1 +/- 0.69
SW480	1.5 +/- 0.12
HT29	1.9 +/- 0.68
DLD-1	2.9 +/- 0.58
LOVO	2.1 +/- 0.25
HCT-116	2.7 +/- 0.46

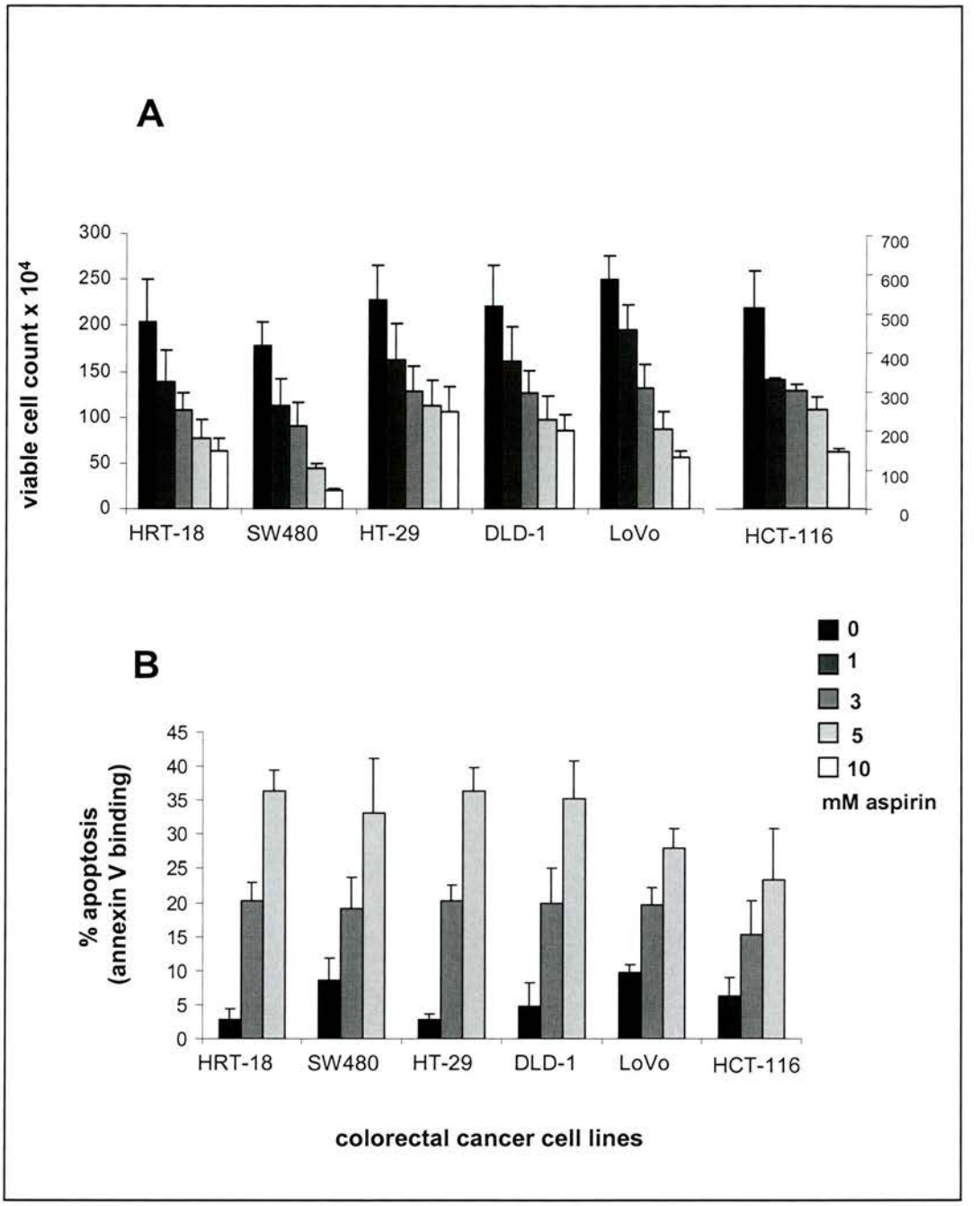

Figure 4.1 Effect of aspirin on cell viability and apoptosis in colorectal cancer cell lines. Aspirin treatment (0-10mM) for 24 hours induces a concentration-dependant decrease in viable cell number in all colorectal cancer cell lines (**A**). All colorectal cancer cell lines undergo apoptosis after aspirin treatment (0-5 mM) (**B**).

Next, it was important to establish whether the reduction in viable cell number that was observed in each of the colorectal cancer cell lines was due to induction of programmed cell death. Annexin-V binding of phosphatidylserine residues externalised during apoptosis was used to determine the proportion of cells undergoing programmed cell death in response to increasing aspirin concentrations. Aspirin treatment induced a concentration-dependent increase in apoptosis in all six of the colorectal cancer cell lines studied, confirming that induction of apoptosis is responsible for the observed reduction in cell viability (Figure 4.1B).

4.3.1.2 Aspirin induces IκBα degradation and p65 nuclear translocation in colorectal cancer cells

The previous work indicated that NFκB nuclear translocation is a key component of aspirin-induced apoptosis in colorectal cancer cells (Stark *et al.*, 2001b). The effect of aspirin on cytoplasmic levels of IκBα, was investigated using immunoblot analysis. Aspirin treatment resulted in concentration-dependent degradation of IκBα, as indicated by a reduction in cytoplasmic protein levels (Figure 4.2A) in all colorectal cancer cell lines. The IC_{50} values for the colorectal cancer cell lines range from 1.5 to 3.1mM aspirin, demonstrating differential sensitivity to aspirin with respect to the concentration at which 50% of the cells are growth inhibited (Table 4.1). The SW480 and HT-29 cell lines have IC_{50} values at the lower end of the range (1.5 and 1.9mM, respectively) and do undergo IκBα degradation at 1mM, whereas the HRT-18 and DLD-1 cell lines have IC_{50} values of 3.1 and 2.9mM and also exhibit IκBα degradation at 3mM. Hence, there is a close relationship between IC_{50} values and IκBα degradation for the individual colorectal cancer cell lines.

In order for the NFκB pathway to be activated, NFκB must translocate to the nucleus following degradation of IκBα. The effect of aspirin on NFκB was studied using immunofluorescence analysis using an antibody to p65, which is the transcriptionally active subunit of NFκB. In untreated colorectal cancer cells, p65 was primarily located in the cytoplasm as expected (Figure 4.2B, first panel). Following aspirin treatment there was nuclear accumulation of p65 in the colorectal cancer cells (Figure 4.2B, second panel). These results show that aspirin-induced apoptosis following IκBα degradation and NFκB nuclear translocation is a generic effect in colorectal cancer.

It is imperative to know whether aspirin as a chemopreventive would be effective in all colorectal cancer given the genetic heterogeneity of the disease. Defects in genes commonly mutated in colorectal cancer, and known to affect apoptotic pathways, might influence the ability of aspirin to induce apoptosis. Hence, the colorectal cancer cell lines were chosen as they harbour differing mutations in genes dysregulated in colorectal cancer. However, mutation status for *APC*, β-catenin, p53 and DNA mismatch repair genes (Table 2.1) does not appear to influence aspirin-induced apoptosis in colorectal cancer lines, emphasising the relevance of the aspirin NFκB anti-tumour effect to colorectal cancer in general. The effect of mutations in p53 and mismatch repair on the NFκB apoptotic response is specifically investigated in Chapter 6.

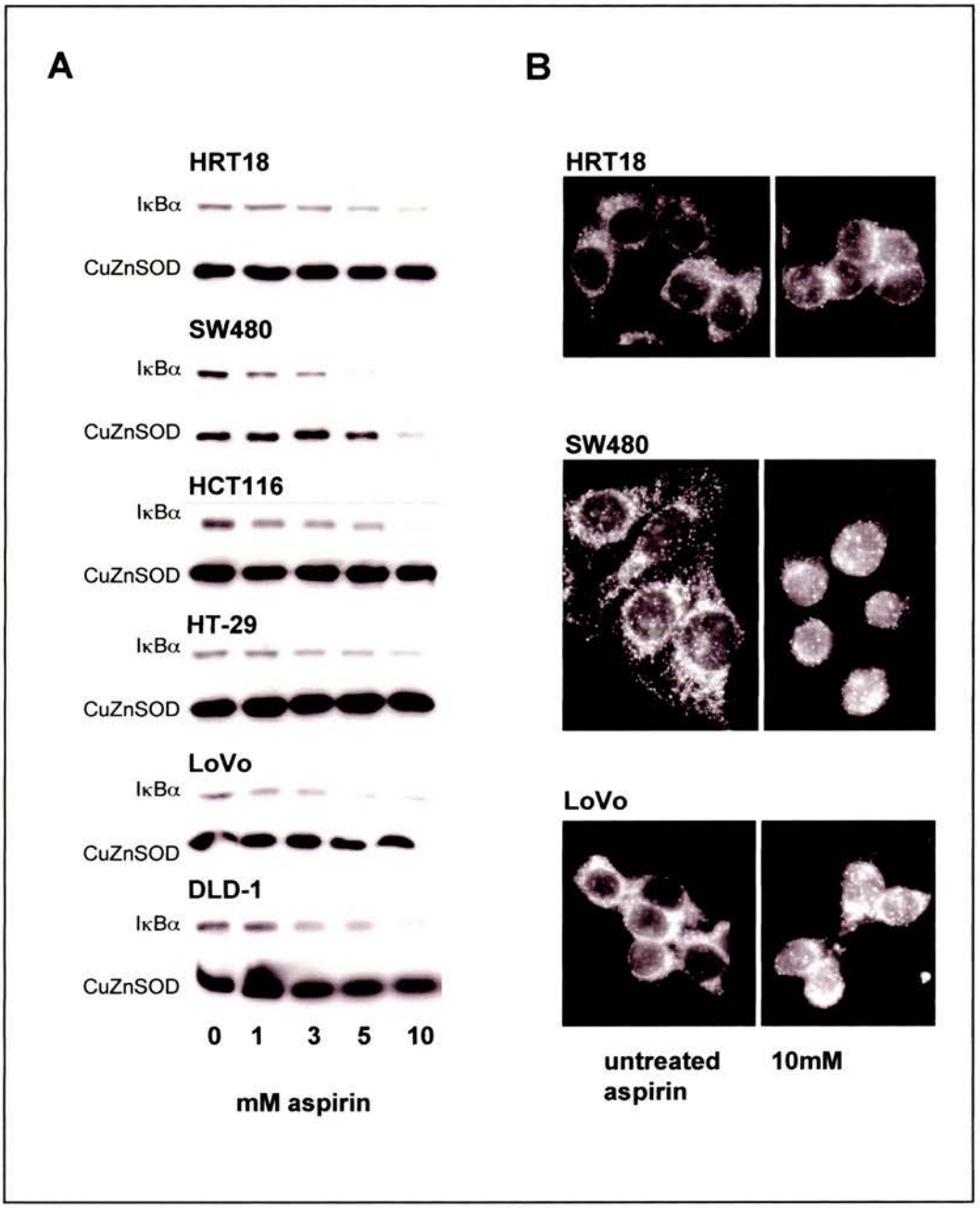

Figure 4.2 Aspirin-induces IκBα degradation and p65 nuclear translocation in colorectal cancer cell lines. Aspirin (0-10mM) induces IκBα degradation in a concentration-dependent manner in the colorectal cancer cell lines (**A**). The Western blots shown are representative of at least 3 independent experiments and copper zinc SOD was used as a control for protein loading. Micrographs (63x) of immunocytochemically stained cells show that aspirin treatment (10mM) induces nuclear accumulation of p65 in the colorectal cancer cell lines (**B**).

4.3.2 Specificity of the aspirin-induced NFκB apoptotic response

4.3.2.1 Aspirin does not induce apoptosis in non-colorectal cancer cells

The next aim was to determine whether the aspirin-induced NFκB apoptotic response was a general anti-tumour effect that was occurring irrespective of cancer type. Hence, the effect of aspirin on the growth of a panel of colorectal cancer cell lines was compared to cell lines derived from other cancer types: breast (MCF-7, MDA-MB-231, T47D), ovarian (A2780), and endometrial (HEC-1-A). The non-colorectal cancer cell lines were chosen based on epidemiological data where there is some evidence to suggest a protective effect in breast cancer and less so in ovarian and endometrial cancer.

The panel of non-colorectal cancer cell lines was treated for 24 hours with aspirin at concentrations of 1, 3, 5 and 10 mM and viable cell number determined by haemocytometric counts. In contrast to the colorectal cancer cell lines, there was no demonstrable effect of aspirin on the viability of the non-colorectal cancer cell lines MCF-7, MDA-MB-231, A2780 and HEC-1-A (Figure 4.3A). Furthermore, there was no concentration-dependent increase in apoptosis in the non-colorectal cancer cell lines following aspirin treatment, which was consistent with the lack of effect on cell viability (Figure 4.3B). Interestingly, although the T47D breast cancer cells did exhibit a concentration-dependent reduction in cell viability, this effect was not as pronounced as that seen in colorectal cancer cells at low aspirin concentrations and furthermore, there was no increase in apoptosis in this cell line. This suggests that T47D may be susceptible

to aspirin in terms of cell viability but it is not due to the same mechanisms, including apoptosis.

To confirm that the non-colorectal cancer cells were less sensitive to apoptosis, three non-colorectal cancer cell lines (two breast and one ovarian) and one colorectal cancer cell line (SW480) were treated with aspirin for a longer time period of 72 hours. Indeed, the non-colorectal cancer cell lines were far less susceptible to apoptosis compared to the colorectal cancer cell line despite treatment with aspirin for 72 h (Figure 4.3C). These findings demonstrate that the anti-tumour activity of aspirin has a substantial degree of specificity for colorectal cancer cells *in vitro*, reflecting the epidemiological evidence for a greater protective effect against colorectal cancer compared to other cancer types.

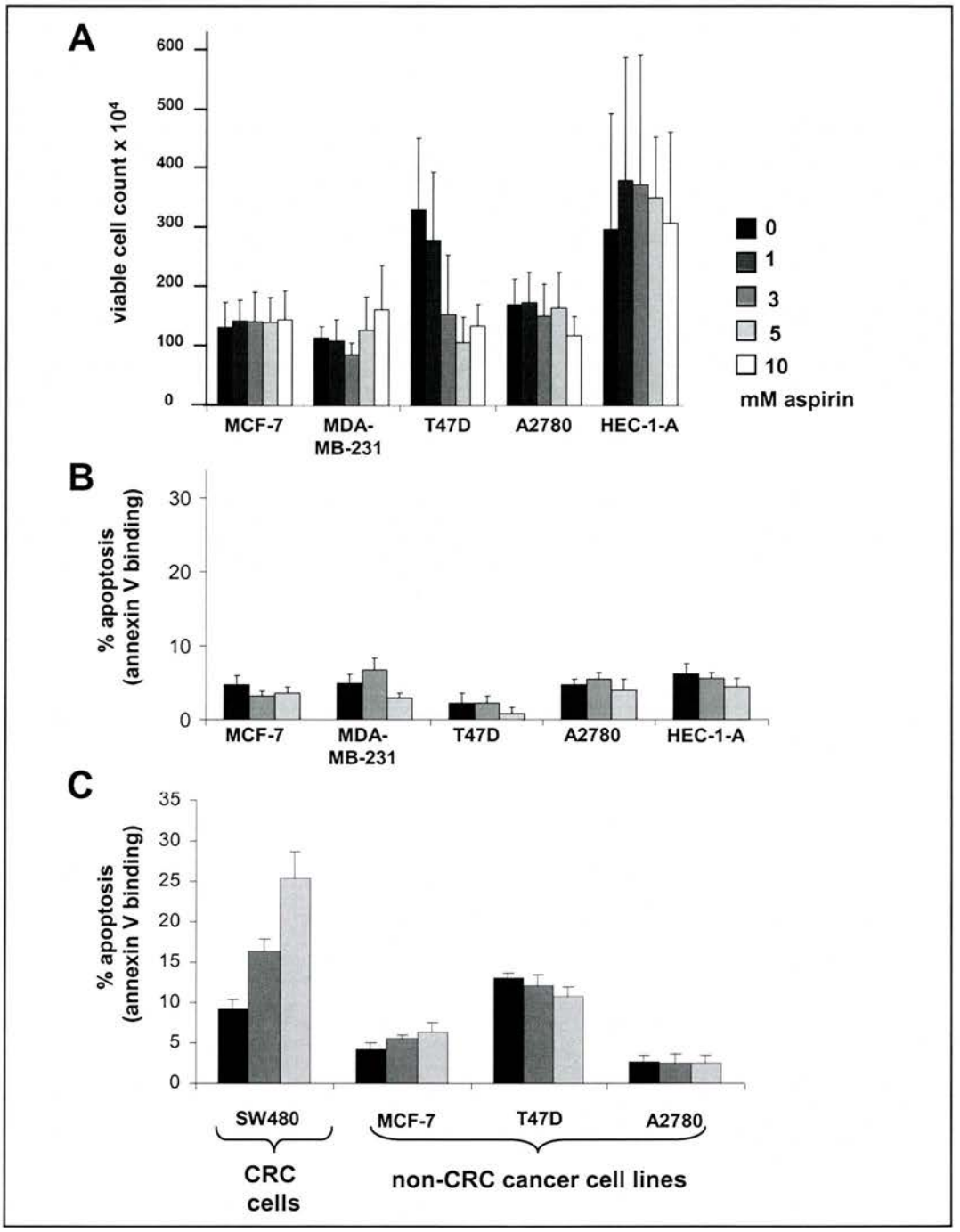

Figure 4.3 Aspirin does not induce apoptosis in non-colorectal cancer cell lines. Aspirin treatment (0-10mM) for 24 hours *did not* consistently affect viable cell number in the non-colorectal cancer cell lines (**A**). There was no increase in apoptosis after exposure for 24 hours (**B**) and 72 hours in the non-colorectal cancer cell lines (**C**).

4.3.2.2 Aspirin's lack of effects on apoptosis in non-colorectal cancer cells are paralleled by failure to activate the NFκB pathway

Variations in cell viability and apoptosis, observed between colorectal cancer and non-colorectal cancer cell lines, may be attributable to differing responses of the NFκB pathway to aspirin. Indeed, in contrast to the colorectal cancer lines, which all underwent concentration-dependent IκBα degradation, there was no change in IκBα after aspirin in any non-colorectal cancer cell line even at the highest dose of 10mM (Figure 4.4A).

Since these findings suggested a cell-type specific NFκB response to aspirin, it was important to determine if the disparate IκBα response was paralleled by a differential effect on NFκB nuclear translocation in the non-colorectal cancer cell lines. Immunofluorescence analysis showed that p65 was situated in the cytoplasm in untreated cells; similar to untreated colorectal cancer cells (Figure 4.4B, first panel). However, in keeping with the observation that there was no IκBα degradation in the non-colorectal cancer cells, aspirin treatment did not induce nuclear translocation of p65 in any of these cell lines (Figure 4.4B, second panel). These data provide evidence of a link between the NFκB response and apoptosis following aspirin exposure. The results establish that the disparity in viability following exposure to aspirin in colorectal cancer cell lines compared to lines derived from other cancer types is associated with markedly differing responses of the NFκB pathway to aspirin. This work suggests that the effect of aspirin on NFκB signalling may be implicated in the differential sensitivity of cancer types to aspirin-induced apoptosis.

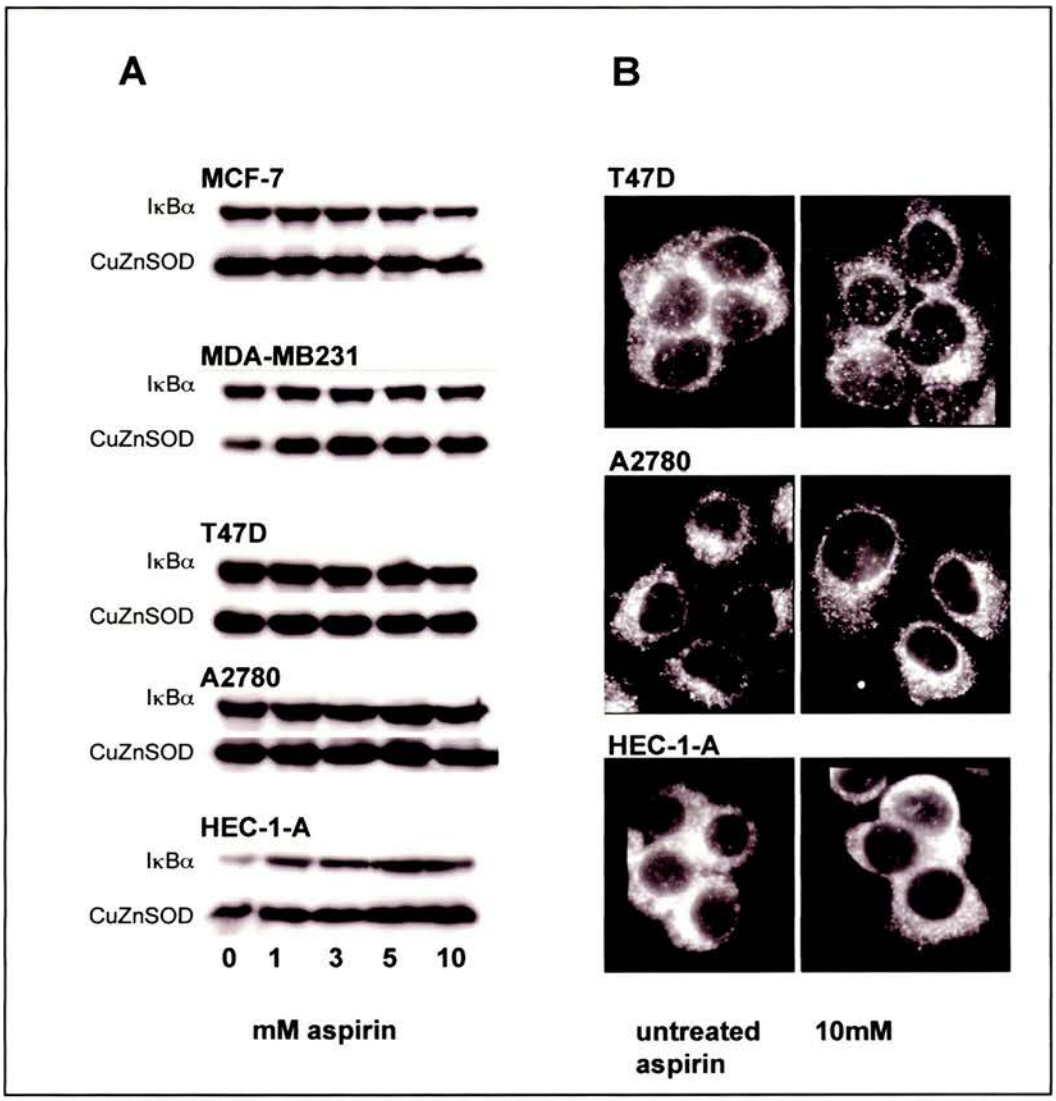

Figure 4.4 Aspirin has *no effect* on NFκB signalling in non-colorectal cancer cell lines. Aspirin (0-10mM) does not induce IκBα degradation in the non-colorectal cancer cell lines (**A**). The Western blots shown are representative of at least 3 independent experiments and copper zinc SOD was used as a control for protein loading. Consistent with the lack of aspirin-induced IκBα degradation , there is no nuclear translocation of p65 upon aspirin treatment, as seen in micrographs (63 x) of immunocytochemically stained non-colorectal cancer cells (**B**).

4.3.2.3 Basal IκBα and p65 protein levels and aspirin-induced apoptosis in colorectal cancer cell lines

High basal NFκB activity and aberrant IκBα expression have been observed in a number of cancers including colorectal cancer (Rayet and Gelinas, 1999). In view of the findings of a cell-type specific NFκB and death response to aspirin, the basal levels of IκBα and p65 were examined to investigate whether they might determine increased sensitivity to apoptosis, and so could be potential molecular markers of response. Immunoblot analysis of cytoplasmic extracts was used to examine basal levels of IκBα and p65 in both the colorectal cancer and non-colorectal cancer cell lines (Figure 4.5). There was no detectable difference in expression of IκBα or p65 or their relative levels (analysed by densitometry) between colorectal and non- colorectal cancer cells that could account for increased sensitivity to apoptosis. These results suggest that sensitivity to aspirin-induced apoptosis is not related to the cytoplasmic pool of either protein available for stimulation.

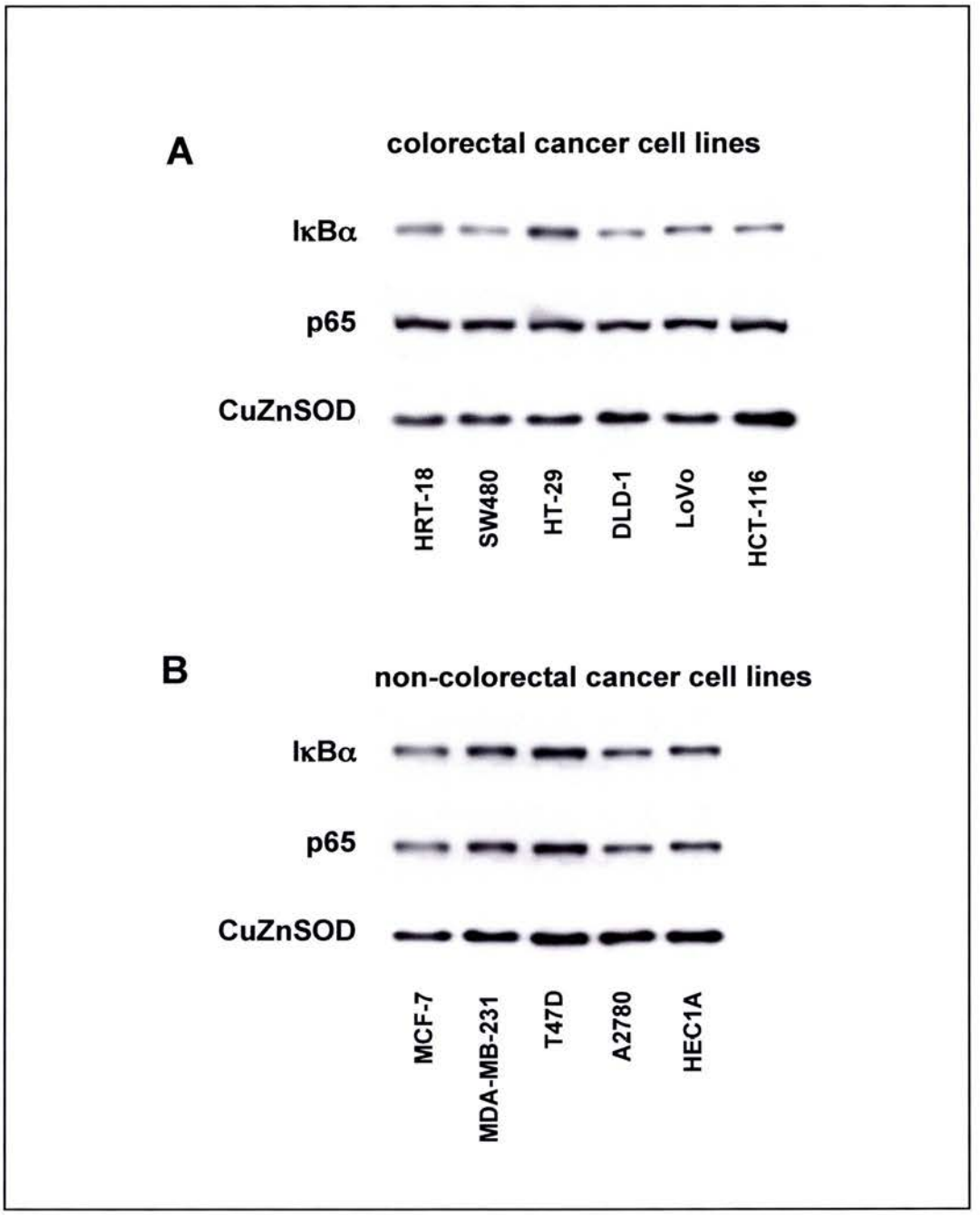

Figure 4.5 Basal levels of cytoplasmic IκBα and p65 proteins in cancer cell lines. Cytoplasmic extracts made from untreated cells & Western blots probed with IκBα and p65 antibody in colorectal cancer cell lines (**A**) and non-colorectal cancer cell lines (**B**). The western blots shown are representative of at least 3 independent experiments and copper zinc SOD was used as a control for protein loading.

4.3.2.4 Aspirin induces IκBα degradation in explants of HT-29 xenografts

To further investigate the specificity of the aspirin response, HT-29 tumour xenografts and other organs including breast, lung, liver, pancreas, spleen, small and large intestine were harvested from mice. The tumour xenograft and organs were maintained *ex vivo* as short-term explants. The explants were washed in PBS, finely sliced, and incubated for 5 hours in DMEM medium containing carrier control or aspirin, at doses sufficient to expose the bulk of the explant to therapeutic levels. Following 5 hours treatment with aspirin, the tissue fragments were homogenized in lysis buffer, after which protein extraction and Western blotting were performed. The Western blots show that aspirin induces IκBα degradation primarily in the HT-29 tumour xenograft and to a lesser extent in normal colon and pancreas (Figure 4.6), supporting the notion that the effects of aspirin on the NFκB signalling pathway may be tissue specific.

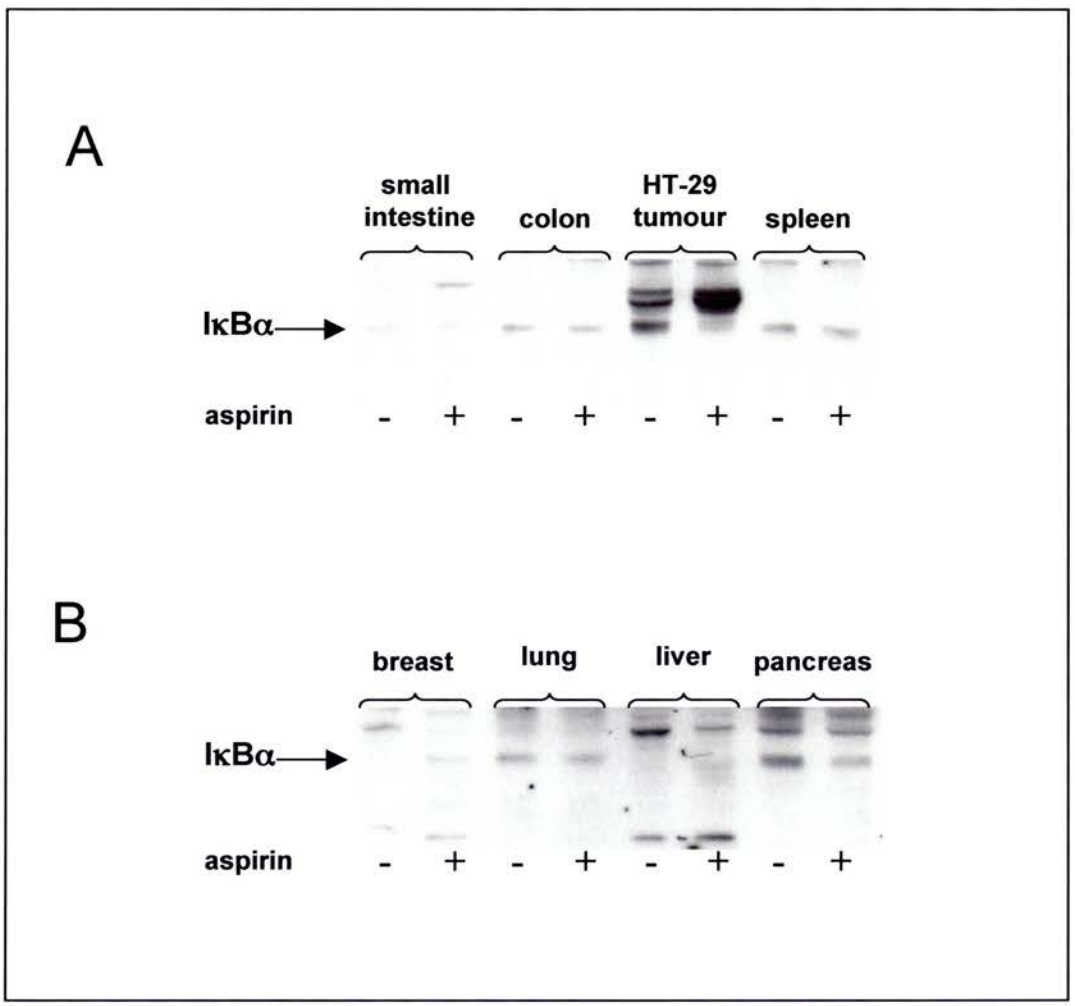

Figure 4.6 Aspirin induces IκBα degradation in explants of HT-29 xenografts.
HT-29 xenografts and control organs (breast, lung, liver, pancreas, small and large intestine, and spleen) were harvested from mice and incubated for 5 hours in DMEM medium containing 10 mM aspirin or carrier control. Western blots of cytoplasmic protein demonstrate IκBα degradation following treatment in the HT-29 tumour xenograft and to a lesser extent in normal colon and pancreas.

4.4 Discussion

The work presented here confirms the importance of the NFκB pathway as a key target for the anti-tumour activity of NSAIDs, and establishes that the effect is generic in colorectal cancer. This is relevant to colorectal cancer chemoprevention aimed at the population at large, which would encompass colorectal cancer of varying genetic backgrounds. In addition, the data demonstrate a striking difference in the response to aspirin, with respect to both cell viability and NFκB signalling, between colorectal cancer cell lines and lines derived from other cancer types. These results show that aspirin-induced apoptosis, associated with IκBα degradation and NFκB nuclear translocation, was restricted to colorectal cancer cells. This relationship between aspirin-induced apoptosis and the effect on NFκB signalling suggests a molecular rationale for the particular sensitivity of colorectal cancer to NSAIDs compared to other cancers.

There is little data directly comparing the anti-tumour effects of NSAIDs *in vitro* between colorectal cancer cells and cancer cells of different tissue origin. A recent study has demonstrated a tissue type-independent effect in prostate, lung, colon, tongue and pancreatic cancer using nitric oxide-donating NSAIDs and although treatment with conventional NSAIDs did have a growth inhibitory effect, it was observed at concentrations in excess of the pharmacologically relevant range after 48 hours of treatment (Kashfi *et al.*, 2002). The data presented in this chapter establish that aspirin has a considerable degree of specificity of apoptotic effect for colorectal cancer cells compared to other cell lines studied, and this reflects the epidemiological observations in the respective tumours. Aspirin induces apoptosis in a panel of colorectal cancer cell lines

but has no effect on viability and apoptosis in cancer cell lines of non-colonic origin. These results contrast with some previous reports of NSAID-induced growth inhibition and apoptosis in breast and endometrial cancer cells (Noguchi *et al.*, 1995; Planchon *et al.*, 1995; Han *et al.*, 1998; Arango *et al.*, 2001), but these differences are reconciled by considering that the majority of these studies used NSAIDs other than aspirin (Noguchi *et al.*, 1995; Planchon *et al.*, 1995; Han *et al.*, 1998), whilst others only observed apoptosis after long exposures (48-96 hours) to high concentrations of salicylate (Sotiriou *et al.*, 1999; Arango *et al.*, 2001). The observation that aspirin decreased cell viability in one of the three breast cancer lines (T47D) is consistent with epidemiological data that suggest a lesser protective effect of NSAIDs against breast cancer. These results clearly indicate important differences between colorectal cancer and other cancer types with respect to aspirin effects on cell viability and apoptosis.

The work here shows that aspirin-induced apoptosis occurs following IκBα degradation and NFκB nuclear translocation, and that this effect is common to all colorectal cancer cell lines studied. Notably, this effect on the NFκB pathway was consistent between colorectal cancer cell lines despite heterogeneity of the lines, with respect to the profile of mutations in *APC*, β-catenin, p53 and DNA mismatch repair genes (Table 2.1). In contrast, aspirin treatment did not induce IκBα degradation or NFκB nuclear translocation in any cell lines derived from cancers of other tissue types, paralleling the lack of consistent changes in cell viability and apoptosis in these lines. It was previously established that the observed effect of aspirin on IκBα and p65 is a *cause* of rather than a consequence of apoptosis, based on the findings that the IκBα degradation was signal-specific and that nuclear translocation of NFκB and apoptosis were blocked by a

dominant negative super-repressor IκBα (Stark *et al.*, 2001b). Hence, the weight of evidence presented here correlating IκBα degradation and p65 nuclear translocation with apoptosis compared to the lack of response in non-colorectal cancer cell lines provides considerable further support for a causal role of the NFκB response as an important component of aspirin-induced apoptosis.

Having shown a striking difference between the colorectal cancer and non-colorectal cancer cell lines, with respect to aspirin effects on NFκB signalling and apoptosis, potential factors that might contribute to the ability of specific cell types to undergo apoptosis were investigated. Increased NFκB activity has been observed in colorectal cancer (Hardwick *et al.*, 2001) and relative resistance to apoptosis has been attributed to high constitutive NFκB activity in other cancers (Bours *et al.*, 1994; Charalambous *et al.*, 2003; Lind *et al.*, 2001). However, there was no evidence that the specificity of the aspirin-NFκB response is related to differential expression of basal IκBα or p65 proteins or their relative expression. The SW480 and HT-29 colorectal cancer cell lines undergo NFκB-mediated apoptosis, despite the considerable difference in basal NFκB activity previously reported between these cell lines (Dejardin *et al.*, 1999).

Aspirin concentrations used here are relevant to pharmacological levels in clinical practice (1-3mM) (Insel P, 1996). Nonetheless, comparisons between cell culture concentrations and serum levels are somewhat artificial because of the inability to accurately mimic *in vivo* metabolism and tissue concentration of the agent in epithelial or tumour cells. Decreased basal levels of apoptosis and hyperproliferative mucosa have

been observed in patients with adenomas, suggesting the existence of a 'field defect' in the colonic mucosa (Anti *et al.*, 2001). Although proportionally less apoptosis was observed at lower concentrations of aspirin, there is evidence that low levels of apoptosis translate into significant tumour regression over time in cell kinetics studies (Pritchard and Watson, 1996). It remains to be determined whether aspirin redresses the balance by inducing apoptosis *de novo* in newly transformed colorectal epithelial cells destined to become malignant clones. There is evidence of NFκB involvement in colonic crypt differentiation and cell turnover in mouse colon, where NFκB activity is greater in proliferating cells at the base of crypts compared to mature cells at the surface (Inan *et al.*, 2000). Thus, it is also possible that the drug corrects deranged mechanisms that permit escape from normal cellular turnover and apoptosis.

In summary, the data presented here demonstrate that there are substantial differences in the anti-tumour effects of aspirin and modulation of NFκB signalling between cancer cells of different tissue origin. The molecular basis of NSAID anti-tumour activity is complex, and these findings provide further evidence that the effects of aspirin on NFκB signalling have particular relevance to colorectal cancer chemoprevention.

Chapter 5

Investigation of molecular determinants of NFκB response to aspirin

5.1 Introduction

Epidemiological data demonstrate a 40-50% reduction in relative risk of developing colorectal cancer in NSAID users, indicating that there may be subsets of people who are not protected by NSAID ingestion, and hence may not respond to chemopreventive measures. Clearly, successful chemoprevention strategies would need to target those who are most susceptible to the disease and those who would respond to such agents in order to improve the risk benefit ratio. The work described in Chapter 4 establishes the relative specificity for colorectal cancer of aspirin-induced apoptosis following IκBα degradation and nuclear translocation of NFκB. Elucidation of the molecular basis underlying the specificity of aspirin-induced NFκB apoptotic response may shed further light on the mechanism of action. Furthermore, it might be possible to identify potential molecular markers that may predict both response to therapy and prognosis. Such molecular markers may be related to heritable predisposition, environmental modifiers, defined somatic genetic alterations or indeed to the mechanism of action of the agent. In this chapter, cyclooxygenase-2 (COX-2) and β-catenin proteins are investigated as potential markers of specificity and response, as both are known to be dysregulated in colorectal cancer and

to interact with the NFκB signalling pathway, and have been suggested as potential molecular targets.

5.1.1 COX-2 and colorectal cancer

There has been considerable debate regarding the role of COX-2 and protection imparted by NSAIDs in colorectal cancer. The analgesic, anti-pyretic, anti-inflammatory and anti-platelet effects of NSAIDs are attributable to inhibition of the cyclooxygenase enzymes (Vane, 1971). The COX enzymes catalyse the rate-limiting step of prostaglandin synthesis, involving conversion of arachidonic acid to the precursor prostaglandin H_2, which is then converted into other prostaglandins by prostaglandin synthases. There are two isoforms of the COX enzymes and recently a third has been proposed, putatively responsible for the anti-inflammatory effects of paracetamol (Willoughby *et al.*, 2000; Chandrasekharan *et al.*, 2002). Cyclooxygenase-1 (COX-1) is constitutively expressed in most cells under physiological conditions (Seibert *et al.*, 1997) and regulates basal turnover of prostaglandins involved in platelet aggregation, mucosal protection and renal blood flow regulation.

COX-2 is the inducible form of the enzyme, which is not expressed under normal conditions but is induced by a variety of stimuli, including growth factors, mitogens and cytokines (Williams *et al.*, 1999). COX-2 is not expressed by normal colonic mucosa and is upregulated in colorectal adenomas and cancers (Eberhart *et al.*, 1994; Chapple *et al.*, 2000). COX-2 is detectable in polyps as small as 2 mm (Oshima *et al.*, 1996), indicating that COX-2 expression occurs after other initiating events such as mutations in genes

disrupted early in the adenoma-carcinoma sequence (Williams *et al.*, 1997). The anti-tumour function of COX-2 was further investigated when Min mice with *Apc* gene mutations (*Apc* $^{\Delta 716}$) were bred with COX-2 null mice (Oshima *et al.*, 1996). COX-2 gene disruption reduced the number and size of the intestinal polyps in the homozygous COX-2 null offspring, indicating a role for COX-2 in colorectal cancer development. Several mechanisms have been suggested by which increased COX-2 expression may initiate and promote tumour growth including carcinogen activation, promotion of cellular proliferation and angiogenesis (Williams *et al.*, 1997), and these are discussed in more detail in Section 1.2.3. COX-2 inhibition has been shown to play a role in aspirin-mediated cell death and may be involved in the anti-tumour activity of NSAIDs (Boolbol *et al.*, 1996). However, there is a growing body of evidence, which indicates that COX-2 is not the predominant target responsible for the anti-tumour activity of NSAIDs (as discussed in Section 1.2.3.3).

5.1.2 Role of *Wnt* signalling in colorectal cancer

Inactivating mutation of *APC* (Nakamura *et al.*, 1991) or AXIN2 (Liu *et al.*, 2000), or by activating mutation of β-catenin is almost universal in colorectal cancer (Sparks *et al.*, 1998), and results in increased nuclear β-catenin which drives Tcf/Lef-mediated transcription promoting tumorigenesis (Korinek *et al.*, 1997). β-catenin acts as a transcriptional activator of growth promoting genes such as *cyclin D1*, *c-myc* and *PPARδ* (Mann *et al.*, 1999) and *survivin* (Kim *et al.*, 2003). Aspirin been shown to increase apoptosis and decrease β-catenin in polyps in Min mice (Mahmoud *et al.*, 1998b). Sulindac sulfone has been shown to induce apoptosis and decrease β-catenin levels (Thompson *et al.*, 2000), and increase APC mRNA expression in rats with chemically-

induced tumours (Kishimoto *et al.*, 2000). Indomethacin has been shown to decrease nuclear β-catenin in colorectal cancer cell lines (Smith *et al.*, 2000). It has been reported that NSAIDs might act by decreasing transcription of β-catenin/Tcf responsive genes (Dihlmann *et al.*, 2001). Furthermore, it is of interest to note that the β-catenin gene (*CTNNB1*) contains a potential binding site for NFκB and that IκBα and β-catenin undergo degradation by the same ubiquitin-mediated pathway (Winston *et al.*, 1999). Recently, β-catenin has been shown to form a complex with NFκB, resulting in a reduction of NFκB DNA binding, transactivation activity, and target gene expression in colon and breast tumour tissues (Deng *et al.*, 2002). Thus, β-catenin may be involved in transcriptional cross regulation of NFκB.

The aim of this work was to determine any parallels in levels of expression of COX-2 or β-catenin in colorectal cancer and aspirin-induced apoptosis mediated by modulation of NFκB signalling, and hence might provide supporting evidence of the specificity of the effect for colorectal cancer. Basal expression levels of COX-2 and β-catenin proteins were compared between colorectal cancer cell lines that undergo aspirin-induced IκBα degradation, NFκB nuclear translocation and apoptosis and the non-colorectal cancer cell lines that do not respond to aspirin (Chapter 4). Identification of molecular markers of response would lend insight not only to the mechanism of action but towards targeted therapy.

5.2 Overview of methods

Protein extracts were made from the untreated colorectal cancer and non-colorectal cancer cell lines studied in Chapter 4 as described in Section 2.6.1 and proteins electrophoresed as described in Section 2.7. The COX-2 expression status of some of the cell lines has been established previously and is shown in Table 5.1. The Western blots were then probed with antibodies to COX-2 and β-catenin to determine relative basal expression levels. Copper zinc SOD antibody was used as a loading control. Protein levels were quantified using densitometry and expressed as a ratio of either COX-2 or β-catenin to Cu/Zn SOD. The levels of protein expression were then correlated with IC_{50} values of aspirin to investigate whether there was a relationship between the expression of these proteins and susceptibility to aspirin-induced apoptosis via IκBα degradation and NFκB nuclear translocation.

Table 5.1 COX-2 expression in cancer cell lines

Cell line	Protein expression	mRNA expression	PGE_2 production	Reference
Colorectal cancer				
SW480	negative	negative	negative	(Parker *et al.*, 1997)
HT-29	positive	positive	positive	(Parker *et al.*, 1997)
DLD-1	negative	positive	-	(Kutchera *et al.*, 1996;Shao *et al.*, 2000b)
LoVo	positive	positive	positive	(Parker *et al.*, 1997)
HCT-116	negative	positive	-	(Kutchera *et al.*, 1996;Shao *et al.*, 2000b)
Non-colorectal cancer				
MCF-7	negative	-	-	(Liu and Rose, 1996)
MDA-MB-231	positive	-	-	(Liu and Rose, 1996)
A2780	-	-	positive	(Hubbard *et al.*, 1988)
HEC-1-A	-	-	positive	(Tamura *et al.*, 2002)

Table 5.2 APC and β-catenin mutation status of cell lines

Cell line	APC	β-catenin
Colorectal cancer		
HRT-18	mutant	wild-type
SW480	mutant	wild-type
HT-29	mutant	wild-type
DLD-1	mutant	wild-type
LoVo	mutant	wild-type
HCT-116	wild-type	mutant
Non-colorectal cancer		
MCF-7	wild-type	wild-type
MDA-MB231	wild-type	wild-type
T47D	wild-type	wild-type
A2780	wild-type	wild-type
HEC-1-A	unknown	unknown

5.3 Results

5.3.1 Basal COX-2 protein levels do not parallel the NFκB response to aspirin

COX-2 expression might explain the heterogeneity of the aspirin response between the colorectal cancer and non-colorectal cancer cell lines. Immunoblot analysis of cytoplasmic proteins demonstrated considerable variation in basal levels of COX-2 between the colorectal cancer cell lines (Figure 5.1A). For example, SW480, HCT-116, HT-29 and LoVo cell lines differ considerably in COX-2 expression (Table 5.1) and yet all underwent IκBα degradation and apoptosis following aspirin treatment (Chapter 4).

Similarly there was variability of COX-2 levels between the colorectal cancer lines and the non-colorectal cancer lines (Figure 5.1B). In addition, there was no demonstrable association between level of COX-2 expression and IC_{50} values. Thus, there was no relationship between basal levels of COX-2 expression and sensitivity to aspirin-induced apoptosis. This provides further support for the notion that COX-independent mechanisms play an important role in the anti-tumour effect of NSAIDs.

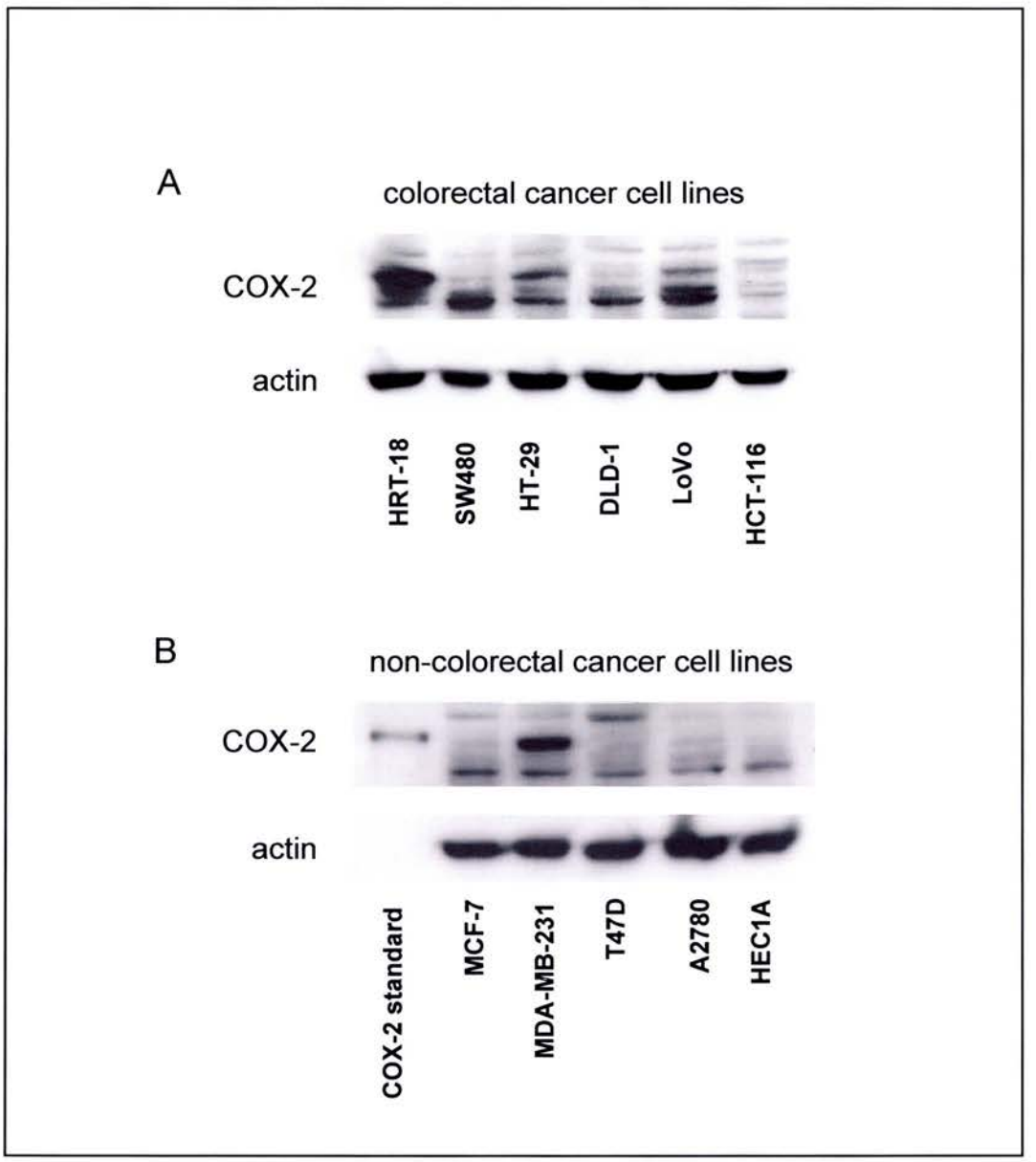

Figure 5.1 Basal COX-2 expression in cancer cell lines.
Cytoplasmic extracts made from untreated cells and Western blots probed with COX-2 antibody in colorectal cancer cell lines (**A**) and non-colorectal cancer cell lines (**B**). The COX-2 standard supplied with the antibody indicates the appropriate band. The Western blots shown are representative of at least 3 independent experiments and actin was used as a control for protein loading.

5.3.2 NFκB apoptotic response to aspirin is independent of basal β-catenin expression

The *APC* and β-catenin mutation status of the colorectal and the non-colorectal cancer cell lines studied in Chapter 4 is outlined in Table 5.2. The mutation status of the colorectal cancer cell lines that respond and those that do not, non-colorectal cancer cell lines, suggest that the anti-tumour effects of aspirin are independent of the type of mutation responsible for increased expression of β-catenin. Next, the relationship between β-catenin and response to aspirin was investigated by immunoblot analysis (Figure 5.2). Within the cell line panels, three colorectal (DLD-1, LoVo, HCT-116) and one non-colorectal (T47D) cancer cell lines expressed relatively higher levels of β-catenin protein. The variable expression of basal β-catenin within both the colorectal and non-colorectal cancer cell lines strongly suggests that β-catenin protein expression itself is not the basis for increased sensitivity to NSAID-mediated apoptosis in colorectal cancer.

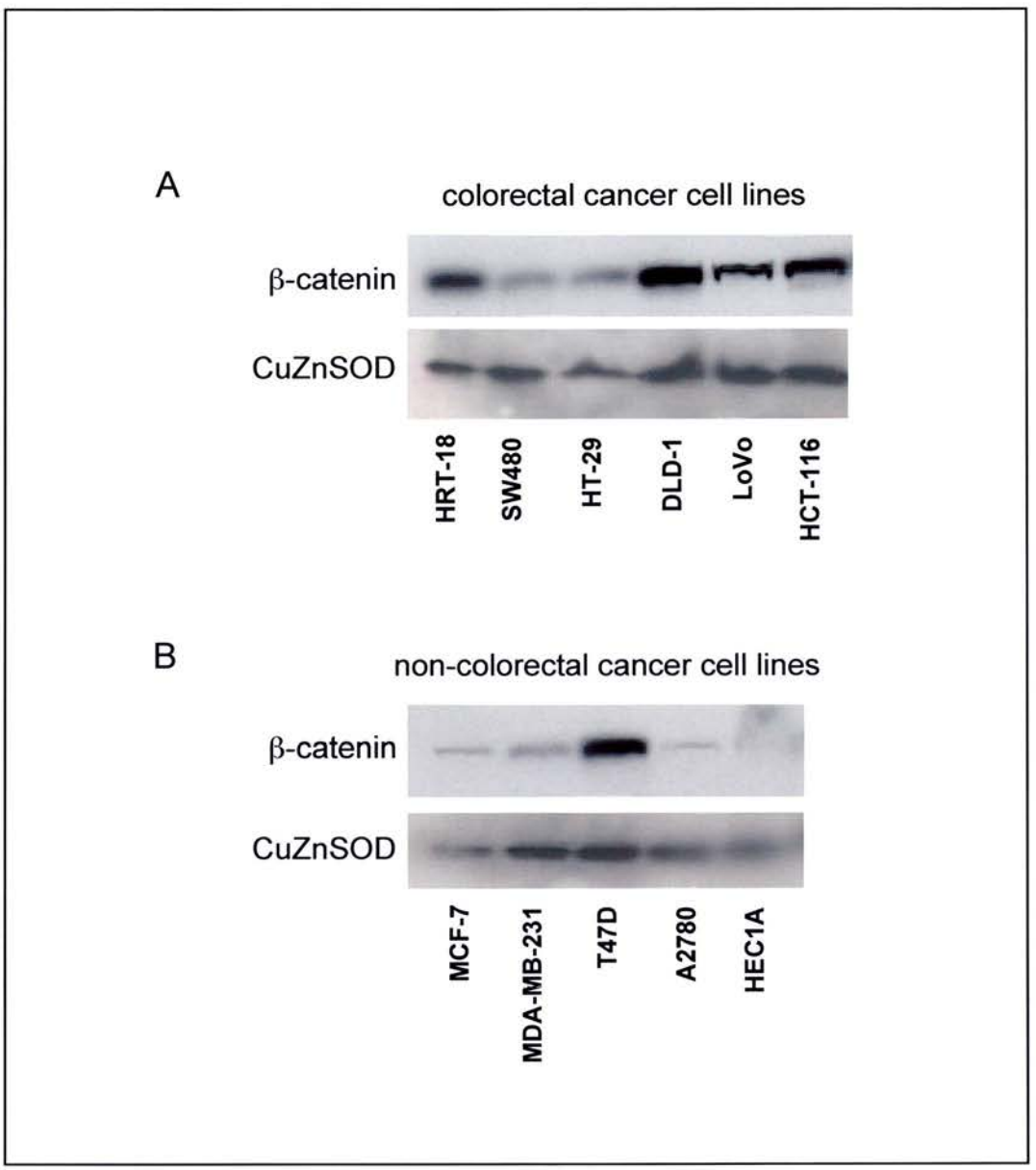

Figure 5.2 Basal β-catenin expression in cancer cell lines.
Cytoplasmic extracts made from untreated cells & Western blots probed with β-catenin antibody in colorectal cancer cell lines (**A**) and non-colorectal cancer cell lines (**B**). The Western blots shown are representative of at least 3 independent experiments and copper zinc SOD was used as a control for protein loading.

5.4 Discussion

Epidemiological data clearly indicate that the anti-tumour effect of aspirin appears to be greater in colorectal cancer compared to cancers from other tissue types. There has been little work thus far investigating the molecular nature of the specificity of this effect. The work presented in Chapter 4 shows that aspirin-mediated apoptosis via IκBα degradation and nuclear translocation of NFκB occurs in colorectal cancer cell lines but not in cancer cell lines of non-colonic origin (Din *et al.*, 2004). The mechanism of action involving apoptosis following modulation of NFκB signalling appears to be relatively specific for colorectal cancer. However, the basal levels of p65 and IκBα proteins do not appear to be conferring increased susceptibility to aspirin-mediated apoptosis (Chapter 4). It is important to investigate the molecular basis of this relative specificity, as it would shed further light on the mechanism of action and may lead to identification of biomarkers that predict resistance to chemopreventive agents.

A recent study examined adenomas that develop in FAP patients whilst on treatment with sulindac in order to identify molecular alterations that may be related to resistance to sulindac (Keller *et al.*, 2001). Immunohistochemical analysis revealed no differences in p53, Bcl-2 and loss of heterozygosity at the *APC* locus between adenomas that were responsive, and those that did not respond to sulindac. However, adenomas resistant to sulindac displayed less loss of membranous β-catenin and less nuclear accumulation of β-catenin. There was no change in stromal COX-2 expression but there was less epithelial COX-2 expression compared to adenomas removed prior to sulindac treatment. The authors concluded that their findings may be due to COX-2 and β-catenin being related to

the underlying mechanism of NSAID action or may indicate the genotype of resistant adenomas. However, since NSAIDs have been shown to decrease both COX-2 and β-catenin *in vitro* and *in vivo,* it is not possible to conclude that these differences are the cause of sulindac resistance as they may be due to the treatment itself.

There is substantial rationale for investigating COX-2 as a potential molecular determinant of response, in view of its role in colorectal cancer development and as a pharmacological target for NSAIDs. Additionally, the promoter region of the human *COX-2* gene has NFκB consensus sites (Appleby *et al.*, 1994), and NFκB has been shown to regulate *COX-2* gene transcription (Schmedtje, Jr. *et al.*, 1997). Recent evidence indicates that COX-2 activity and resultant production of prostaglandins can function both as positive as well as negative regulators of NFκB (Poligone and Baldwin, 2001) suggesting a degree of cross-regulation between COX-2 and NFκB.

With respect to specificity, COX-2 expression is more common in 69% of endometrial cancers and 89% of ovarian serous cystadenocarcinomas compared with normal tissue (Landen, Jr. *et al.*, 2003). Additionally, it has been reported that the inconclusive nature of an NSAID effect in epidemiological data in breast cancer might be related to the observation that only a subset of breast cancers express COX-2 (Howe *et al.*, 2001b). In the work presented here, there was no relationship between COX-2 protein levels and apoptotic response to aspirin in any cell type. Furthermore, there was considerable variation in COX-2 expression within the colorectal cancer cell lines, which were all susceptible to aspirin-induced apoptosis. Expression of COX-2 is regulated at several levels including transcriptional and post-transcriptional regulation via stabilisation of

COX-2 mRNA and COX-2 degradation (Shao *et al.*, 2000b). Both the SW480 and HT-29 colorectal cancer cell lines undergo NFκB-mediated apoptosis, despite striking differences COX-2 mRNA and protein expression between these cell lines (Table 5.1). The magnitude of the apoptotic response in the colorectal cancer cell lines did not correlate with the levels of COX-2 expression. This work contributes to the mounting persuasive evidence that COX-2-independent as well as COX-2-dependent mechanisms play a role in the anti-tumour effects of NSAIDs (Rigas and Shiff, 2000).

In view of the well-characterised dysregulation of *Wnt* signalling in colorectal cancer compared to other cancers, β-catenin was investigated as a potential molecular determinant of response to aspirin. The colorectal cancer cell lines studied here have known *APC* or β-catenin mutations (Table 5.2) and it can be observed that aspirin-mediated apoptosis occurs irrespective of the type of mutation responsible for the upregulation of β-catenin. The differing expression of basal β-catenin within both the colorectal and non-colorectal cancer cell lines indicates that β-catenin protein expression itself does not govern sensitivity to aspirin-induced apoptosis via NFκB nuclear translocation. These results indicate that aspirin-induced apoptosis secondary to IκBα degradation and NFκB nuclear translocation occur irrespective of early mutational events in colorectal cancer.

Although investigation of COX-2 and β-catenin, in the context of the cell lines studied, has not identified either as potential molecular determinant of the NFκB apoptotic response to aspirin; these results are nonetheless relevant to NSAID-mediated

chemoprevention. Chemoprevention is highly relevant those with a hereditary predisposition to colorectal cancer where the risk benefit ratio of any chemopreventive agent would be reduced. Patients with FAP have mutations of the tumour suppressor gene *APC* increasing their risk of colorectal cancer and such mutations also occur in 85% of patients with sporadic colorectal cancers (Powell *et al.*, 1992). Mutations in *APC* result in increased levels of nuclear β catenin, which drives transcription of growth promoting genes. NSAIDs have been shown to induce apoptosis both in murine models of FAP (Boolbol *et al.*, 1996; Mahmoud *et al.*, 1998b) and in patients with FAP (Pasricha *et al.*, 1995; Keller *et al.*, 1999;Stoner *et al.*, 1999).

In addition to investigation of candidate molecular markers of response to aspirin, strategies aimed at systematic identification of putative molecular markers should be undertaken. In this respect, a bioinformatics-based approach to identify differential expression of genes in colorectal cancer compared to other cancers, especially where there is little evidence of a chemopreventive effect of NSAIDs, may be useful. These candidate molecular markers may then be investigated further with respect to interactions with the NFκB signalling pathway to provide mechanistic insight.

Chapter 6

Influence of mismatch repair and p53 signalling on NFκB response to aspirin

6.1 Introduction

Research efforts are focussed at both the clinical level to determine dose and duration of usage and also the molecular level, to fully delineate the mechanisms involved in NSAID anti-tumour activity and to inform novel drug design. It is also important to determine whether there are subsets of colorectal cancers that are more or less susceptible to NSAID-mediated chemoprevention, as this will add insight at a mechanistic level and for rational development of clinical trials.

The work in Chapter 3 showed that NFκB plays a pivotal role in aspirin-mediated apoptosis in colorectal cancer cells (Stark *et al.*, 2001b). NFκB has been shown to have both pro- and anti-apoptotic effects (Barkett and Gilmore, 1999). Such disparate effects are due to differences in stimuli, NFκB composition, cell-type and distinct κB binding specificities of individual complexes resulting in diverse target gene specificity (Epinat and Gilmore, 1999). The relative specificity of this NFκB-dependent effect for colorectal cancer compared to other cancer cell types was described in Chapter 4 (Din *et al.*, 2004).

However, it is also important to determine whether there is heterogeneity within colorectal cancer with respect to the NFκB-dependent apoptotic response.

It is well established that genomic instability, by increasing mutational load, promotes neoplastic progression in colorectal cancer. The p53 tumour suppressor gene is involved in cell cycle control, apoptosis and maintenance of genomic stability and is frequently somatically mutated in colorectal tumours, heralding malignant transformation (Baker *et al.*, 1989; Baker *et al.*, 1990; Honma *et al.*, 2000). Another important contributor to genomic instability is defective DNA MMR resulting in microsatellite instability (MSI) (Veigl *et al.*, 1998). MSI is the hallmark of tumours arising in HNPCC and is also found in 15% of sporadic colorectal cancers (Boland *et al.*, 1998; Brown *et al.*, 1998). The majority of MSI tumours are due to germline mutations in *hMLH1, hMLH2* and *hMSH6* genes in familial cases, and due to *hMLH1* promoter hypermethylation in sporadic cancers (Kuismanen *et al.*, 2000). The result of such defects is rapid accumulation of mutations in growth-regulatory genes such as *BAX, TCF4,* and *TGFβRII* contributing to accelerated tumorigenesis (Peltomaki, 2001). Although an increased rate of p53 mutations would be expected, there is in fact an inverse relationship between p53 mutations and severity of microsatellite instability (Cottu *et al.*, 1996; Samowitz *et al.*, 2001).

There is considerable rationale for studying the effect of p53 and MMR on the aspirin NFκB response. Firstly, it is important to define the generality of the response with respect to p53 and to elucidate its effectiveness in terms of late mutational events in colorectal cancer. Secondly, the influence of MMR on the NFκB response should be investigated given that clinical trials of polyp prevention using aspirin in HNPCC carriers

are already underway (CAPP-2, 2005). There is evidence that p53 signalling and DNA MMR are molecular targets for NSAIDs (Ruschoff *et al.*, 1998; Shao *et al.*, 2000a; Goel *et al.*, 2003), suggesting that the anti-tumour effect may, in part, involve countering the effects of genetic instability in colorectal cancer. In addition, there is evidence of interaction between p53 and NFκB (Wu and Lozano, 1994) through regulatory interdependence involving competition for common co-activators (Webster and Perkins, 1999). Genetic aberrations in tumours have also been shown to be involved in determining response to chemotherapeutic agents (O'Connor *et al.*, 1997; Weller, 1998; Ribic *et al.*, 2003).

In light of the importance of p53 and MMR in colorectal cancer and given that genomic instability can influence response to chemotherapeutics, it is highly relevant to determine the p53 and hMLH1 dependency of the effects of aspirin on NFκB signalling.

6.2 Overview of methods

An HCT-116 colorectal cancer cell line model was used to definitively address the role of p53 and MMR status in the NFκB-dependent apoptotic response to aspirin (Table 6.1).

Table 6.1 HCT-116 cell line characteristics

Colorectal cancer cell line	MMR mutation status	p53 function
HCT-116	hMLH1 deficient	wild-type
HCT-116 $^{+ch3}$	hMLH1 proficient	wild-type
HCT-116 $^{p53-/-}$	hMLH1 deficient	p53 null

The HCT-116 cell line has a homozygous mutation in *hMLH1* resulting in a truncated, non-functional protein (Boyer *et al.*, 1995). The HCT-116 sub-line where *hMLH1* expression is restored by chromosome 3 transfer (HCT-116^{+ch3}) is competent in DNA MMR (Koi *et al.*, 1994). The p53 null HCT-116 sub-line (HCT-116 $^{p53-/-}$) was created by targeted homologous recombination (Bunz *et al.*, 1999). All three cell lines were maintained and treated with aspirin (1,3,5 and 10mM) or carrier control (at same concentrations as aspirin) as described in Sections 2.2 and 2.3. After aspirin treatment, cell viability and apoptosis were assessed by haematocytometric counting and annexin V assays described in Sections 2.4 and 2.5. Cytoplasmic protein extracts were made (Section 2.6.1.1) and Western blotting was used to determine cytoplasmic IκBα (Section 2.7). NFκB nuclear translocation was studied using immunocytochemistry (Section 2.9). For transfection experiments, cell lines were transiently transfected with the 3enhancer-ConA NFκB-dependent luciferase reporter construct in which transcription of the firefly luciferase gene is driven by 3 κB binding sites (Roff *et al.*, 1996) as described in Section 2.10.

6.3 Results

6.3.1 p53 and hMLH1 expression and basal expression of IκBα and p65 proteins in colorectal cancer cell lines

The best model to specifically address whether p53 and hMLH1 influence the aspirin-induced NFκB apoptotic response is by using HCT-116 colorectal cancer cell lines that differ with respect to the specific genetic change under investigation. The protein levels of p53 and hMLH1 were determined in each cell line. This confirmed that hMLH1 was not expressed in the parental HCT-116 cell line but was expressed in the HCT-116 $^{+ch3}$ cell line (Figure 6.1A). As expected there was no p53 protein detected in the HCT-116 $^{p53-/-}$ cell line, but p53 was expressed in the parental HCT-116 cell line which has wild-type p53 (Figure 6.1A). It was first important to consider whether p53 and hMLH1 mutation status might affect basal levels of IκBα and p65 proteins. Immunoblot analysis of untreated cytoplasmic extracts demonstrated that basal expression of IκBα and p65 was similar in each HCT-116 cell line genotype (Figure 6.1B). These results indicate that changes in MMR mutation status and p53 expression do not affect the cytoplasmic pool of IκBα or p65 proteins. Basal levels of NFκB DNA binding were examined to determine whether constitutive NFκB DNA binding is affected by changes in p53 or MMR status. EMSAs performed on nuclear extracts of untreated cells showed basal NFκB DNA binding in the three colorectal cancer cell lines (Figure 6.1 C). The differences in basal NFκB DNA complexes in the HCT-116 $^{+ch3}$ and HCT-116 $^{p53-/-}$ cell lines compared to the parental HCT-116 cell line were marginal.

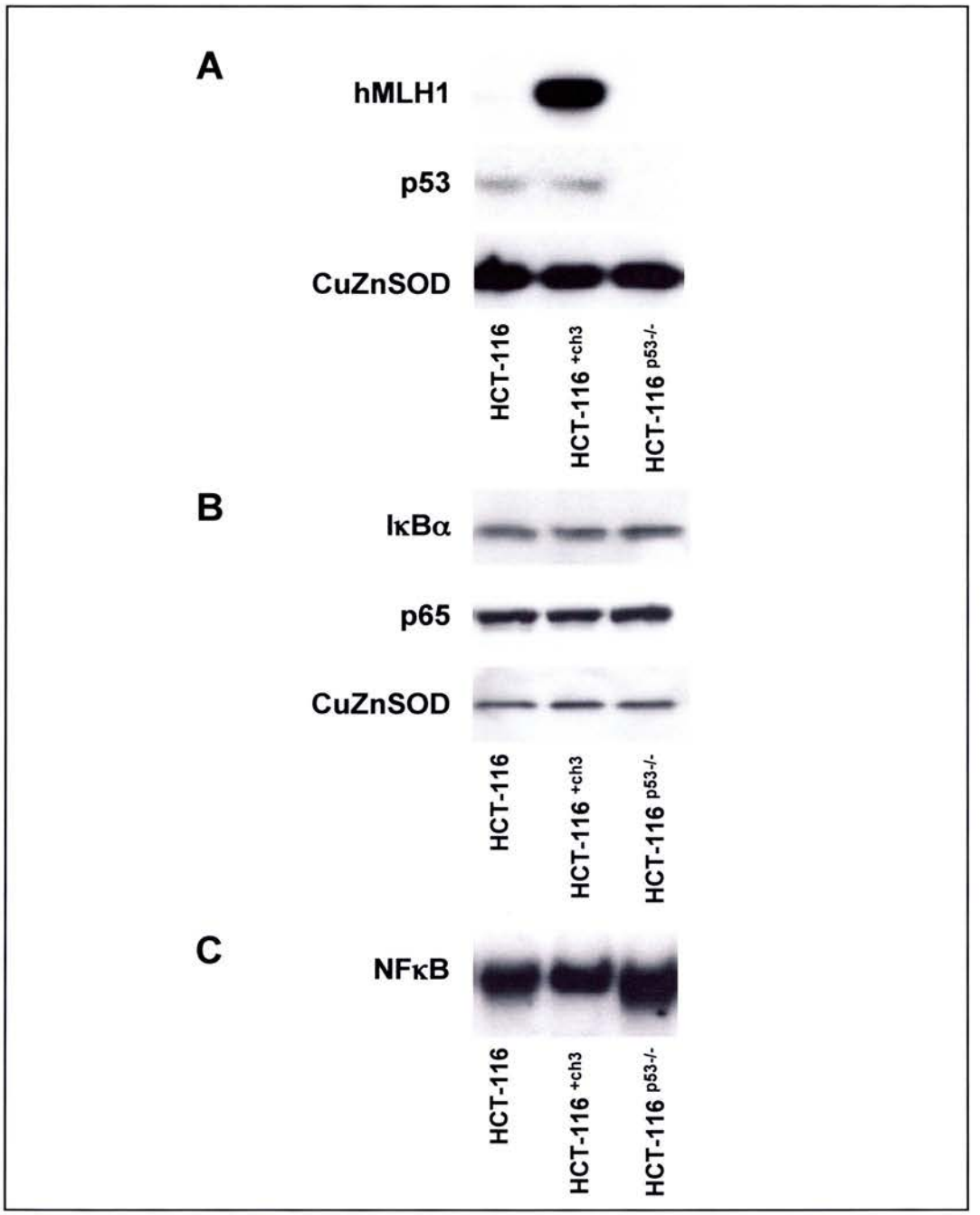

Figure 6.1 Basal expression of p53, hMLH1, IκBα & p65 in untreated HCT-116 cell lines. Cytoplasmic extracts made from untreated cells & Western blots probed with p53 & hMLH1 antibodies to confirm expression profile (**A**). Cytoplasmic extracts were probed with IκBα & p65 antibodies to examine basal expression (**B**). The Western blots shown are representative of at least 3 independent experiments and copper zinc SOD was used as a loading control. EMSA using NFκB consensus oligonucleotide demonstrated basal NFκB DNA binding in nuclear extracts(**C**).

6.3.2 Aspirin induces apoptosis in colorectal cancer cell lines independent of p53 and MMR status

To determine the p53 and MMR dependence of the effects of aspirin on cell viability, the HCT-116 $^{+ch3}$ (wild-type p53 and hMLH1 proficient) and HCT-116 $^{p53-/-}$ (p53 null and hMLH1 deficient) cell lines were compared to the parental HCT-116 (wild-type p53 and hMLH1 deficient) colorectal cancer cell line. In triplicate dose-response experiments, all colorectal cancer cell lines were treated for 16 hours with aspirin (1, 3, 5 and 10 mM) and viable cell number determined by haemocytometric counts. Aspirin treatment resulted in a concentration-dependent decrease in the number of viable cells in all three colorectal cancer cell lines (Figure 6.2A). Furthermore, the cell lines showed proportionate decreases in cell viability at each concentration increment, indicating a similar pattern of response to aspirin, irrespective of p53 or MMR status (Figure 6.2B). The IC_{50} values were calculated from the growth curves of the aspirin-treated colorectal cancer cell lines (Table 6.2), and there were no significant differences in levels of cell death between cell line genotypes (Student's t-test).

Table 6.2 IC_{50} values for colorectal cancer cell lines

CRC cell line	IC_{50}	Fold increase apoptosis	
		3mM aspirin	5 mM aspirin
HCT-116	2.8 +/- 0.26	2.3	3.1
HCT-116 $^{+ch3}$	3.1 +/- 1.6	2.4	2.6
HCT-116 $^{p53-/-}$	4.3 +/- 0.82	1.5	1.8

It was next confirmed that the reduction in viable cell number in each of the cell lines was indeed due to apoptosis for all three genotypes, and not simply a growth inhibitory effect. Following aspirin treatment, cells were stained with Annexin V, which binds phosphatidylserine residues that are externalised during apoptosis and thus serves as a marker for programmed cell death. Consistent with the reduction in cell viability, aspirin induced a concentration-dependent increase in the proportion of apoptotic cells in all three colorectal cancer cell lines (Figure 6.2C). There was no significant difference in apoptotic response between any of the HCT-116, HCT-116 $^{+ch3}$ and HCT-116 $^{p53-/-}$ cell lines. These data suggest that aspirin induces apoptosis in colorectal cancer cells and that it is independent of p53 and MMR status.

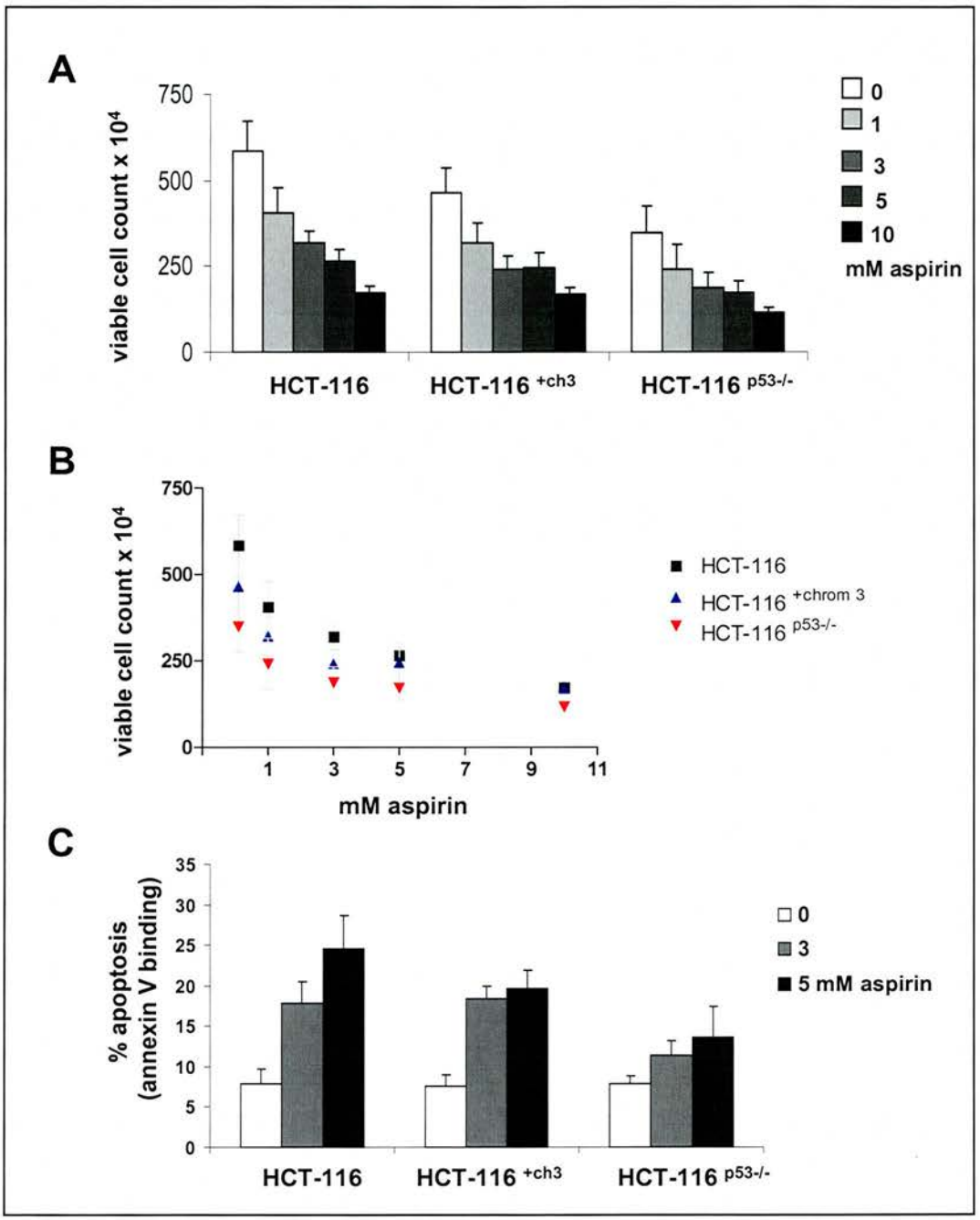

Figure 6.2 Effect of aspirin on cell viability and apoptosis in HCT-116 cell lines. Aspirin treatment (0-10mM) induces a concentration-dependant decrease in viable cell number in all cell lines (**A**). The decrease in cell viability is proportionate at each concentration increment indicating a similar pattern of response in each cell line (**B**). Annexin V assay used to determine that all cell lines undergo apoptosis after aspirin treatment (0-5 mM) (**C**).

6.3.3 Aspirin induces IκBα degradation and NFκB nuclear translocation in colorectal cancer irrespective of MMR and p53 status

Having shown that all three colorectal cancer cell lines undergo aspirin-induced apoptosis irrespective of MMR and p53 status, the effect of aspirin on NFκB signalling was studied in each cell line. The previous work (Chapter 3 & 4) showed that aspirin induces IκBα degradation, permitting NFκB to migrate to the nucleus and that the NFκB nuclear translocation was essential for aspirin-induced apoptosis. Aspirin treatment induced equivalent degradation of IκBα in a concentration-dependent manner in HCT-116, HCT-116 $^{+ch3}$ and HCT-116 $^{p53-/-}$ lines (Figure 6.3). Next, it was determined whether aspirin-induced IκBα degradation was accompanied by NFκB nuclear translocation in the colorectal cancer cell lines. Immunofluorescence analysis showed that p65, the transcriptionally active subunit of NFκB, was predominantly located in the cytoplasm in all untreated cells (Figure 6.4A). After treatment with aspirin for 16 hours, there was nuclear accumulation of p65 in all three colorectal cancer cell lines irrespective of p53 and MMR status (Figure 6.4B). These findings show that aspirin-induced apoptosis due to modulation of the NFκB pathway occurs irrespective of derangements in p53 signalling and the MMR system.

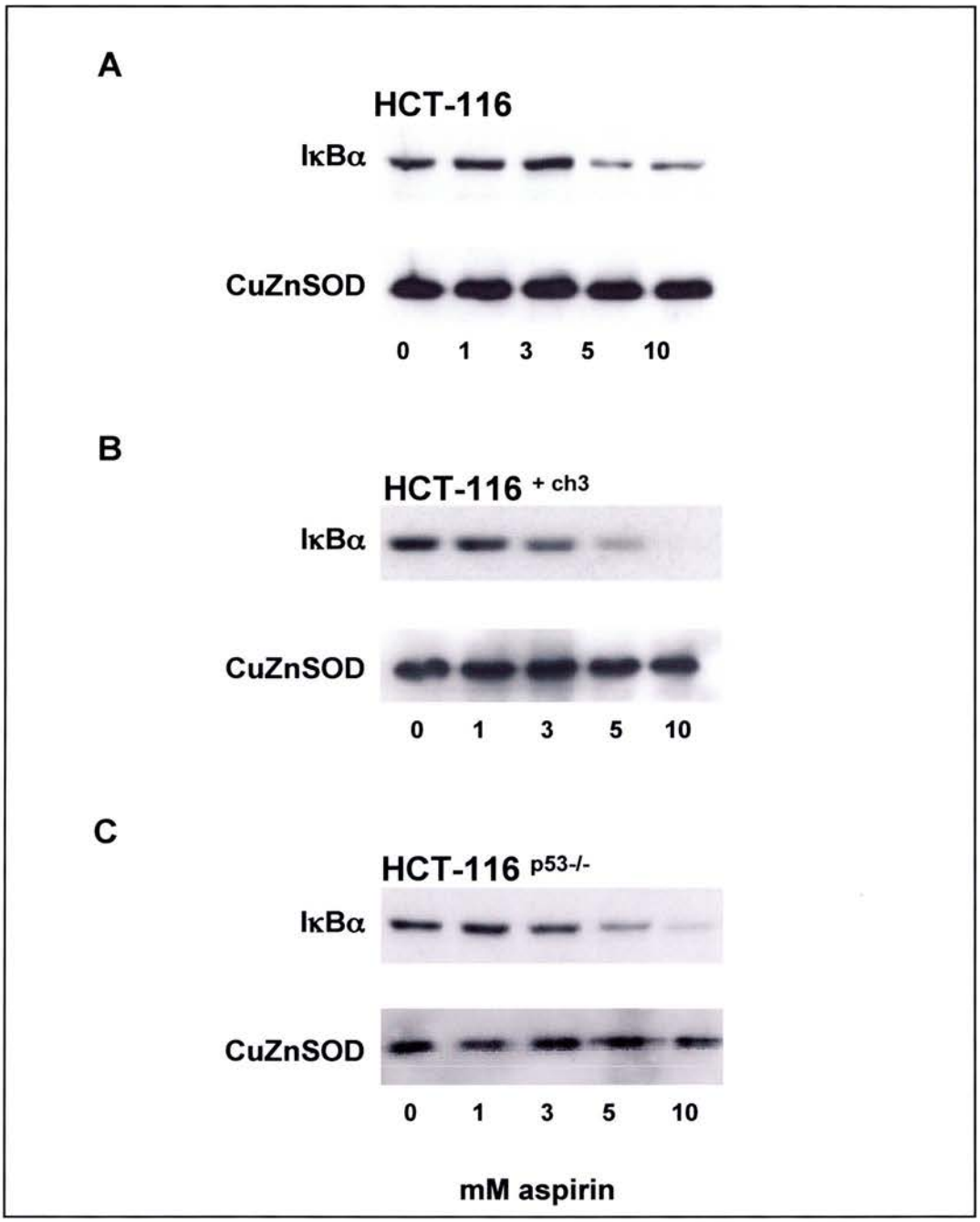

Figure 6.3 Aspirin induces IκBα degradation in HCT-116 cell lines. Following aspirin treatment, cytoplasmic extracts were probed with IκBα antibody. Western blot analysis shows that aspirin treatment (0-10mM) for 16 hours induces IκBα degradation in a concentration-dependent manner in the HCT-116 (**A**), HCT116^{+ch3} (**B**) and HCT-116 $^{p53-/-}$ (**C**) colorectal cancer cell lines.

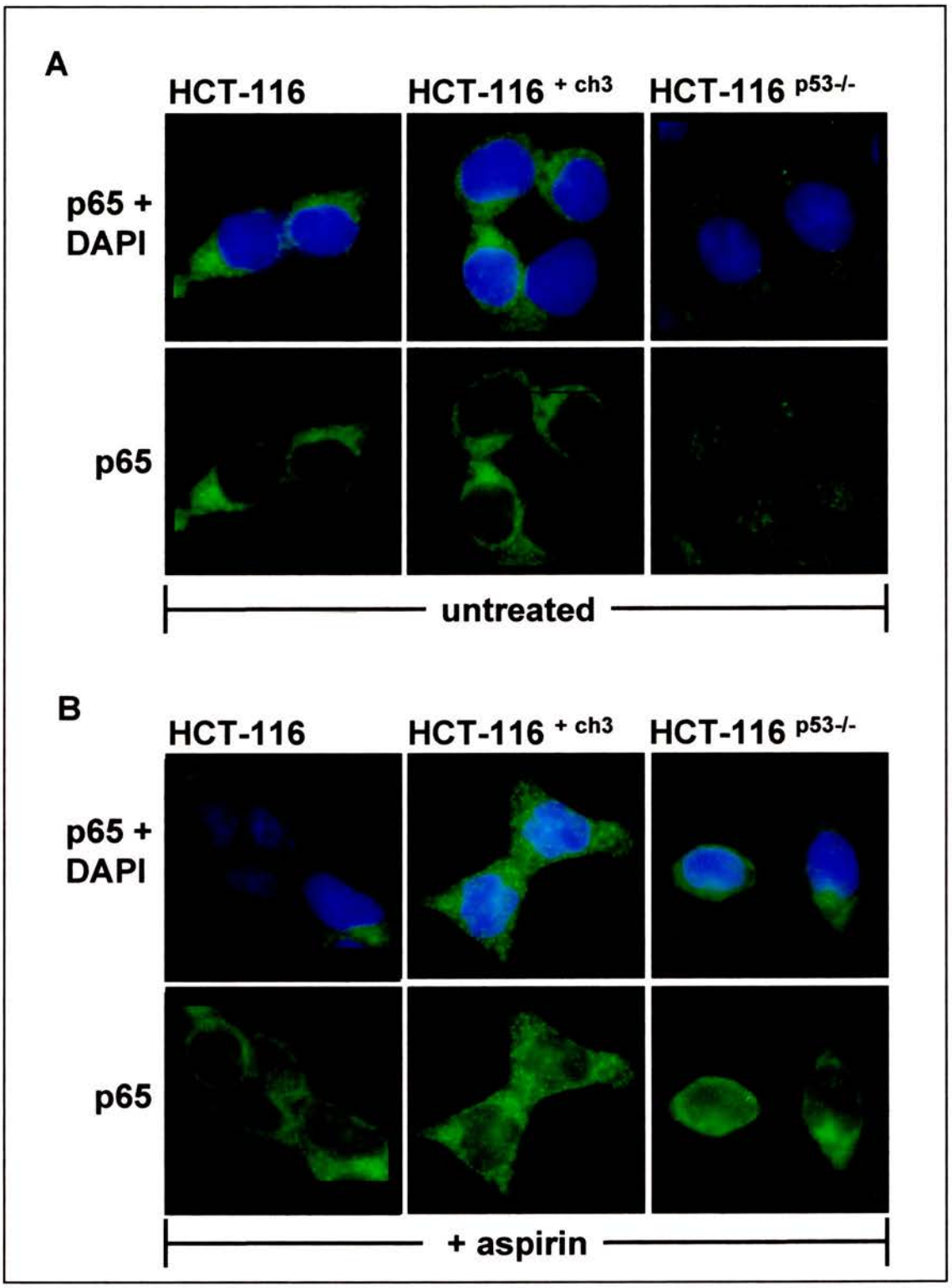

Figure 6.4 Aspirin induces nuclear translocation of p65 HCT-116 cell lines. Micrographs (63 x) of immunocytochemically stained cells show p65 is mainly located in the cytoplasm in untreated cells (**A**). Aspirin treatment (10mM) for 16 hours induces nuclear accumulation of p65 in the HCT-116, HCT116^{+ch3} and HCT-116 $^{p53-/-}$ CRC cell lines. DAPI staining indicates the position of the nucleus.

6.3.4 Aspirin-mediated repression of NFκB driven reporter activity is unrelated to MMR and p53 status

Paradoxical to the understanding of the classical NFκB pathway, aspirin-induced nuclear translocation of NFκB has been shown to *repress* NFκB transcriptional activity in colorectal cancer cell lines (Stark *et al.*, 2000). This work showed that aspirin-induced NFκB nuclear translocation and repression of NFκB-driven transcription, and that this was not a generic inhibitory effect on signalling pathways or transcriptional machinery itself. Studies of the kinetics of the response demonstrated that IκBα degradation and NFκB nuclear translocation precede repression of NFκB-driven transcription, indicating a causal relationship. Furthermore, inhibition of NFκB nuclear translocation by super-repressor IκBα inhibited aspirin-mediated repression of NFκB transcriptional activity. This observation is further supported by the finding that NFκB induced by some cytotoxic stimuli acts as an active *repressor* of anti-apoptotic genes (Campbell *et al.*, 2004).

Hence, the effect of aspirin on NFκB-driven transcription was investigated to determine whether p53 or MMR mutation status affect the ability of aspirin to induce NFκB transcriptional repression in colorectal cancer cells. The cell lines were transiently transfected with the 3enhancer-ConA NFκB-dependent luciferase reporter construct in which transcription of the firefly luciferase gene is driven by 3 κB binding sites (Roff *et al.*, 1996). A reporter plasmid with deleted κB sites served as control. Following transfection, cells were exposed to aspirin for 16hrs. There was substantial repression of

the basal levels of NFκB-driven reporter activity following aspirin exposure. There was a concentration-dependent decrease in NFκB-driven reporter activity in each cell line genotype, irrespective of p53 or MMR status (Figure 6.5). These findings show that p53 and MMR status do not influence aspirin-induced repression of NFκB transcriptional activity, and hence downstream regulation of NFκB target genes.

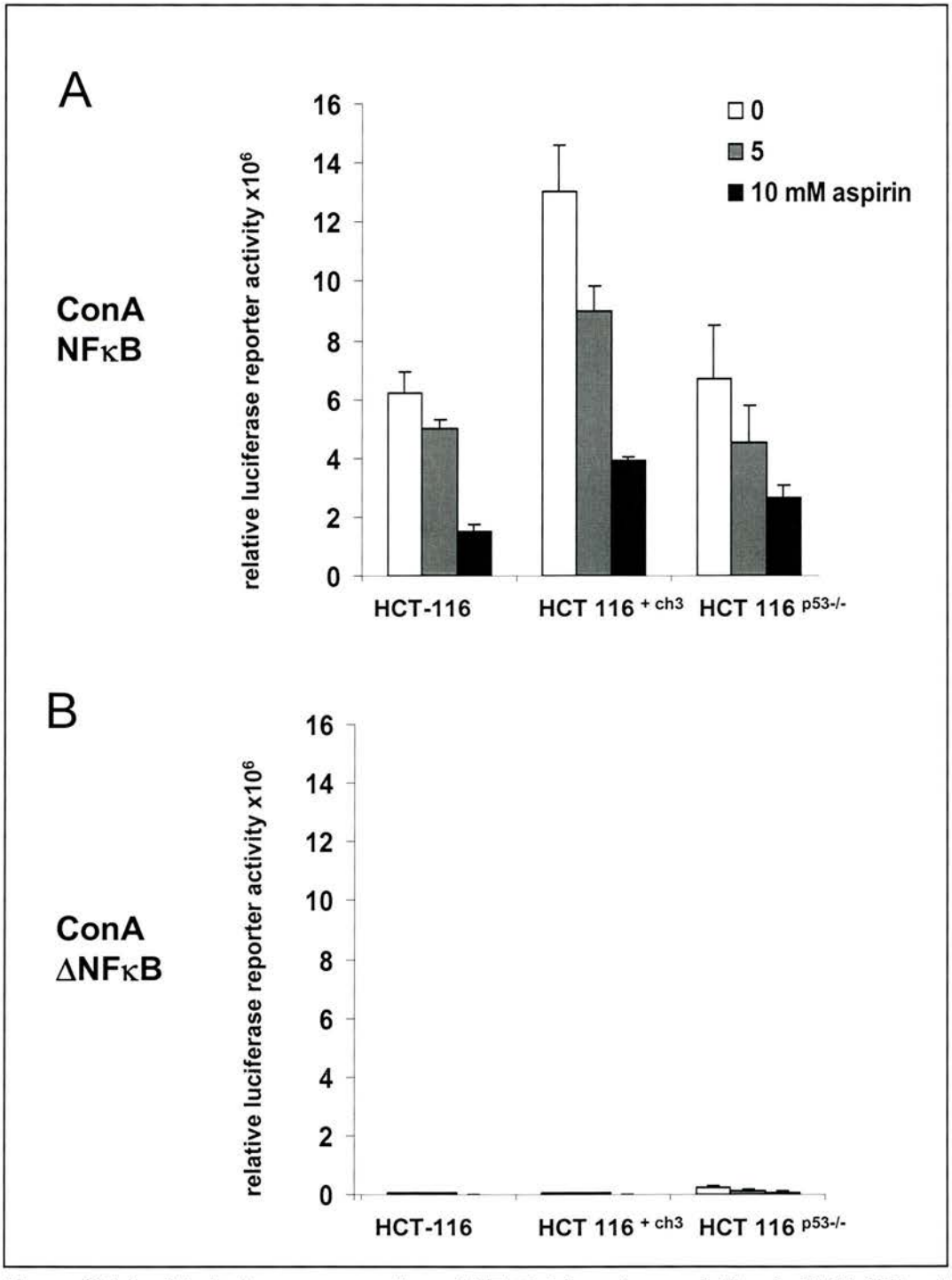

Figure 6.5 Aspirin induces repression of NFκB-driven transcription in HCT-116 cell lines. Cells transfected with ConA NFκB-dependent luciferase reporter, containing 3 κB binding sites (**A**), or equivalent plasmid with κB consensus sites deleted ConA ΔκB (**B**). All cells co-transfected with control CMV-β-galactosidase plasmid. After 16hrs treatment aspirin (0-10 mM), luciferase and β-galactosidase assays were performed on cell lysates and relative luciferase activity calculated.

6.4 Discussion

This work shows that aspirin causes apoptosis and modulates NFκB signalling in a p53 and MMR independent fashion. Furthermore, these results show that aspirin induces nuclear translocation of p65 that is associated with repression of kappa-B driven transcription, again independent of p53 and MMR status. This considerably extends previous observations that aspirin's effects on NFκB signalling are central to aspirin-mediated apoptosis in colorectal cancer cells (Stark *et al.*, 2001a; Din *et al.*, 2004).

It is well established that aspirin has a chemopreventive effect in colorectal cancer but the mechanism of action has not been fully characterised. p53 function and DNA mismatch repair have been proposed as potential targets responsible for the anti-tumorigenic properties of NSAIDs (Ruschoff *et al.*, 1998; Shao *et al.*, 2000a).

The p53 signalling pathway is central to regulating cell growth and death, and stabilisation of p53 by mutation is a key event occurring late in colorectal tumorigenesis (Baker *et al.*, 1990). Several studies have shown an association between p53 mutation status and sensitivity to chemotherapeutic drugs in colorectal and other cancers (O'Connor *et al.*, 1997; Weller, 1998). The p53 pathway has been postulated as a potential target since NSAIDs have been shown to alter levels of p53 (Goldberg *et al.*, 1996; Kralj *et al.*, 2001). Furthermore, cross-talk has been reported between p53 and NFκB through competition for common co-activators (Webster and Perkins, 1999). Indeed, wild-type p53 has been shown to suppress constitutive NFκB activity and lead to apoptosis (Shao *et*

al., 2000a), suggesting that tumours expressing wild-type p53 may be more susceptible to aspirin-induced apoptosis.

Previous data indicated that colorectal cancer cell lines expressing wild-type p53 were not more sensitive to aspirin-induced apoptosis mediated by NFκB signalling, but there were differences other than p53 status between the cell lines studied (Din *et al.*, 2004). In this work, the effects of p53 on the NFκB-induced apoptotic response were specifically investigated by using HCT-116 cells in which the p53 gene has been homozygously disrupted by targeted homologous recombination (Bunz *et al.*, 1999). Using this approach, these findings indicate conclusively that aspirin-induced apoptosis is independent of p53. Furthermore, p53 does not appear to play a role in aspirin-induced effects on NFκB signalling or on the repression of NFκB transcriptional activity. These findings are important in terms of chemoprevention since colorectal cancers with mutant p53 have been shown to differ in behaviour from those expressing wild-type p53, with respect to response to chemotherapeutic agents and prognosis (Weller et al., 1998; Bunz et al., 1999).

Defective DNA mismatch repair is charactersitic of HNPCC and around 15% of all colorectal cancers also show genetic instability, mainly due to epigenetic silencing of *hMLH1* but also due to somatic MMR gene defects (Herman *et al.*, 1998). The DNA MMR system has been implicated as a potential pathway for modulation that may contribute to NSAID anti-tumour activity (Ruschoff *et al.*, 1998; Goel *et al.*, 2003). The results in Chapter 4 suggested that MMR-proficient cells may be more sensitive to aspirin-induced apoptosis since the MMR-deficient cell lines had greater IC_{50} values than

MMR-proficient cell lines. Hence, this work specifically examined whether MMR status influenced IκBα degradation-dependent aspirin-induced apoptosis, by comparing the MMR-deficient HCT-116 cell line to its proficient counterpart HCT-116^{+ch3}. There was a dose-dependent increase in apoptosis after 16 hours treatment with aspirin that paralleled IκBα degradation, NFκB nuclear translocation and repression of NFκB-driven transcription. Indeed the HCT-116 cells have a marginally lower IC_{50} value and show a greater fold increase in apoptosis when compared to the MMR-proficient cell line but this was not significant (Table 6.2). Although there were no significant differences detected in the aspirin-NFκB apoptotic response attributable to MMR status, long-term *in vitro* aspirin exposure has been shown to select for microsatellite stability in colorectal and gastric cancer cell lines (Ruschoff *et al.*, 1998; Yamamoto *et al.*, 1999a). It has also been shown that aspirin treatment increased MMR protein expression and apoptosis in colorectal cancer cell lines (Goel *et al.*, 2003). Despite substantial rationale for study of the influence of MMR on the aspirin-induced NFκB apoptotic response, there was no evidence for MMR dependency of these effects, suggesting that the MMR system is not the predominant pathway responsible for NSAID-mediated anti-tumour activity.

This work consolidates previous findings that aspirin-induced apoptosis occurs after IκBα degradation, NFκB nuclear translocation and repression of NFκB driven transcription. The results presented here shed further light on the complex mechanisms by which NSAIDs induce cell death in colorectal cancer. Elucidation of the mechanism lies in defining the relative contribution of putative targets to aspirin's anti-tumour activity. There was no evidence to suggest the involvement of p53 or DNA mismatch repair on inducing the NFκB pathway, nor on the ensuing apoptotic response following aspirin.

Genomic instability due to p53 or MMR dysfunction has been to shown to be associated with resistance to chemotherapeutic agents. Hence, these findings have relevance to rational design of novel therapeutics. In addition, since the effects of aspirin on NFκB and apoptosis occur in cancers arising from different genetic backgrounds, these findings have clinical relevance when considering design of chemoprevention trials not only in genetically predisposed individuals with defective MMR, but also in the general population and also since p53 mutational events are important during development and progression of colorectal neoplasia.

Chapter 7

Clinical studies of the effects of NSAIDs on NFκB signalling

7.1 Introduction

The majority of patients with colorectal cancer present with late stage disease and 50% will die from metastatic disease. Novel approaches aimed at early detection, prevention and treatment of colorectal cancer are being pursued to reduce the considerable associated morbidity and mortality, and prolong survival. Although patients with more advanced disease may be offered chemotherapy, it is not possible to predict who will benefit from the survival advantage, and this has to be offset against potential treatment-related toxicity. Identification of molecular and genetic events responsible for colorectal carcinogenesis has advanced rapidly over the last three decades. Conventional chemotherapy works on the basis that malignant cells divide more rapidly than normal cells. Novel therapies are being sought that target specific molecular pathways that may underlie the development of colorectal cancer. Despite extensive molecular and genetic characterisation of the disease, not all findings have translated into clinical benefit for colorectal cancer patients. Translational studies form the first step towards bridging the gap between the molecular advances and progress in terms of colorectal cancer survival.

NSAIDs have shown much promise as potential chemopreventive agents based on epidemiological studies. Numerous mechanisms of action have been investigated *in vitro* using cell culture and animal models, which then need to be confirmed in a clinical setting. In the previous work, aspirin has been shown to induce apoptosis in colorectal cancer cells through modulation of the NFκB pathway. Aspirin induces degradation of the inhibitory protein IκBα and allows NFκB to translocate to the nucleus where it can regulate target gene transcription. Aspirin also induced IκBα degradation in short-term explants from HT-29 tumour xenografts and colorectal cancer patients (Stark *et al.*, 2001b; Din *et al.*, 2004). Although, the *ex-vivo* explant experiments suggested that aspirin is able to modulate the NFκB pathway in tissue specimens, the aspirin concentrations were used to generate a biological effect and therefore the clinical relevance is unclear. Having demonstrated this mechanism of aspirin's action *in vitro*, it was next important to establish the relevance of these findings to the clinical scenario in patients with colorectal cancer. Hence, the next step was to conduct a clinical study in patients to establish whether the molecular observations of aspirin's effects on NFκB occur *in vivo,* at pharmacologically attainable concentrations of aspirin.

The aim of this work was to establish whether the modulatory effect of NSAIDs on NFκB signalling could be detected in pilot clinical study protocols *in vivo,* to demonstrate the translational potential of this mechanism of action in colorectal cancer patients. Hence, the work presented in this chapter was aimed specifically at assessing whether reduction in IκBα and induction of apoptosis, observed *in vitro*, could be demonstrated in pilot studies in patients.

Patients with rectal cancer recruited to the study were treated with aspirin or the COX-2 selective inhibitor rofecoxib for 1 week. The goal of any potential chemopreventive agent is to achieve a low risk to benefit ratio and those at greatest risk, such as patients with HNPCC or FAP, may be the group who would benefit most from chemoprevention. In addition, it is important to establish whether any effects of aspirin occur at earlier stages in development of colorectal cancer. Therefore, patients with HNPCC or FAP were recruited to the study. Biopsies were taken from normal rectal mucosa, rectal tumour and polyps (if present) before and after treatment and examined for evidence of NFκB modulation and apoptosis. This is the first study to examine the *in vivo* effects of NSAIDs on NFκB signalling in patients. There are clearly limitations of clinical studies employing molecular endpoints, as well as practical difficulties in execution of such research to minimise the effect of biological variability and sampling artefacts. Nonetheless, the clinical research presented here shows that studies involving repeated sigmoidoscopy and biopsy are feasible and acceptable. Furthermore, there is reason to be optimistic since the pilot data show tentative evidence that future studies of aspirin can detect real differences once the level of variability has been defined.

7.2 Overview of Methods

Three study groups were assembled: patients with established rectal cancer and genetically predisposed patients with HNPCC or FAP. The inclusion criteria were patients over the age of 18. The exclusion criteria were patients already taking NSAIDs or anti-coagulants, allergy, asthma, pregnancy, peptic ulcer disease, bleeding disorder and previous stroke.

The dosing regimens were selected on pragmatic grounds and comprised COX-2 selective inhibitor rofecoxib 25mg OD or aspirin 300-600mg OD or 600mg QDS. The higher (analgesic) dose of aspirin is designed to maximise any biological effect, while the lower dose will allow determination of the clinical relevance for future chemoprevention, when the lower dose would be more practical.

All studies had local ethical, management and MHRA approval (LREC numbers 99/5/8,99/5/21,99/5/50 and CTA Number 17844/0001/001), which were written and applied for by myself. Molecular endpoints are changes in the level of apoptosis, IκBα degradation, NFκB nuclear translocation and expression of downstream NFκB driven genes. This work is in progress and the results presented here are on IκBα degradation and apoptosis. Given the short duration of treatment there was no expectation of a clinically detectable effect.

Briefly, after informed consent biopsies were taken from normal rectal mucosa and rectal tumour (if present) before and after 7 days of NSAID treatment. Immediately, part of the

biopsy specimen was fixed in formalin for paraffin embedding and sectioning for H&E staining. Another part of the biopsy was snap-frozen in liquid nitrogen for protein analysis. Cytoplasmic and nuclear proteins were extracted as described in Section 2.6.2. Western blots of cytoplasmic proteins were used to assess the levels of IκBα. Apoptotic counts in H&E stained sections were performed by Dr Angus McGregor as described in Section 2.5.3.

The Quantity One (BioRad) imaging densitometer software was used to quantify the protein levels of IκBα. Repeat biopsies were not taken from patients, nor were patients entered into the study more than once. To assess one aspect of intra-experimental variation, Western blots of the same protein extracts from individual patients were repeated and quantified as a measure of reproducibility. Wilcoxon signed rank test was used to establish whether there were significant differences between pre- and post-treatment IκBα protein levels. Dr Peter Teague, MRC Human Genetics Unit statistician, verified the statistical methods employed. This work presented here is pilot data and the clinical studies are ongoing.

7.3 Results

7.3.1 IκBα expression *in vivo* after NSAIDs

7.3.1.1 Rectal cancer patient study

IκBα levels were determined in biopsies of normal mucosa and rectal tumour before and after aspirin or rofecoxib administration in 16 patients with rectal neoplasia (14 adenocarcinoma, 2 large adenomas). Cytoplasmic protein, extracted from biopsies of normal rectal mucosa and tumour before and after treatment, was analysed by Western blotting for IκBα levels. IκBα band intensity was quantified by densitometry in triplicate and corrected for control protein loading.

Basal IκBα levels in protein extracts from normal mucosa and rectal tumours before treatment with NSAIDs are shown in Figure 7.1. Inter-sample variability was noted in pre-treatment levels of IκBα in extracts from untreated normal mucosa and from rectal tumours. Since upregulation of NFκB signalling has been observed in colorectal cancer (Rayet and Gelinas, 1999), IκBα levels in normal mucosa (n=16) were compared to IκBα in matched rectal tumours (n=16). There were no significant differences in basal IκBα expression prior to NSAID treatment in normal rectal mucosa compared to rectal tumour from the same patient (p=0.27, Wilcoxon signed rank test).

Next, IκBα levels were determined in normal mucosa and rectal tumours before and after treatment with NSAIDs. IκBα protein levels were quantified by densitometry, corrected for control protein loading, and the actual optical density values are shown in Table 7.1.

Table 7.1 IκBα expression following NSAIDs in rectal cancer patients

Patient ID	NSAID Type & Dose	IκBα-normal		Difference between pre & post-NSAID IκBα in normal		IκBα-tumour		Difference between pre & post-NSAID IκBα in tumour	
		Pre*	Post*	% lower	% higher	Pre*	Post*	% lower	% higher
AB	300 mg aspirin OD	0.92	1.06	-	16.1	1.22	1.13	7.3	-
SF		1.16	1.15	1.1	-	1.06	1.09	-	2.9
HG	600 mg aspirin OD	1.70	1.09	35.7	-	1.14	1.10	3.8	-
JS		1.01	0.97	4.1	-	0.91	1.03	-	12.9
MC		1.02	1.16	-	14.4	0.94	0.86	8.2	-
DB	600 mg aspirin QDS	1.15	1.04	9.3	-	1.14	1.05	8.2	-
JC		0.99	0.91	8.0	-	1.08	0.97	10.7	-
JM		0.78	1.01	-	29.7	1.13	0.91	19.7	-
JL		1.01	1.01	-	0.6	0.97	1.02	-	5.1
JP		0.91	1.17	-	28.1	1.24	0.94	24.1	-
ES		1.04	1.01	2.6	-	0.99	0.95	4.3	-
JK	25 mg rofecoxib OD	1.04	0.81	22.8	-	1.04	0.98	5.7	-
KW		1.19	1.44	-	21.4	1.22	0.73	40.4	-
DW		1.04	0.97	6.2	-	1.03	0.95	7.7	-
AW		0.90	0.95	-	5.2	0.73	1.00	-	36.7
GW		1.21	1.17	2.8	-	1.18	1.18	no change	

*- Units are optical density (OD) and number given is the average of repeat density measurements on different gels for each patient

The number of patients that have lower cytoplasmic IκBα band intensity in samples taken after NSAID treatment was 9 (56.25%) in normal mucosa and 11 (68.75%) in rectal

tumours. Representative Western blots from the patients demonstrating lower levels of cytoplasmic IκBα expression after NSAID treatment are shown in Figure 7.2. However, there were 6 patients (37.5%) in whom normal mucosa showed a greater IκBα band intensity, and 4 patients (25%) in whom rectal tumour also showed greater IκBα band intensity in the post-treatment biopsies. One patient had samples that showed no difference in IκBα protein levels in rectal tumour in post- compared to pre-treatment biopsies (Table 7.1). The IκBα levels for patients treated with the higher dose of aspirin (600mg QDS) are shown in Figure 7.3C. There may be a difference in IκBα after treatment in this group but this was not significant when considering multiple testing. Hence, no conclusion can be drawn.

The results show variability in IκBα expression both before and after treatment with NSAIDs. If a pragmatic cut-off of a difference of ≥5% change in IκBα densitometry levels before and after treatment is used, then there are lower IκBα levels in normal mucosa in 6 patients (37.5%) and in rectal tumour in 8 patients (50%). However, there were also greater IκBα levels in normal mucosa and rectal tumours in 6 (37.5%) and 3 (18.75%) patients respectively. These results suggest that there is considerable variability in IκBα protein expression and since there is no placebo group it is not possible to attribute any change in IκBα levels to treatment with NSAIDs. Hence, these pilot studies have exemplified the difficulties of translating laboratory findings to the clinical scenario.

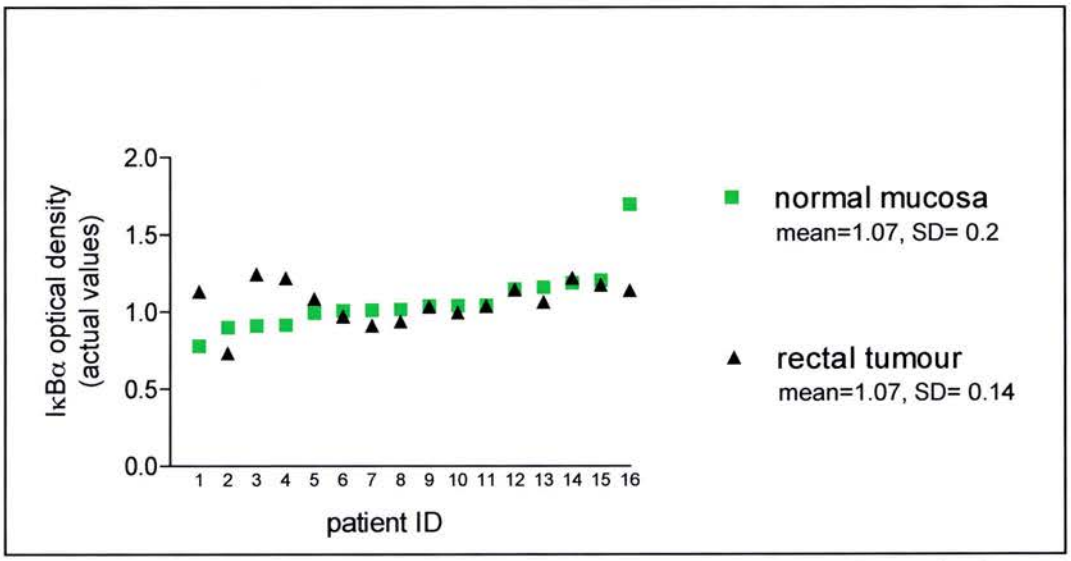

Figure 7.1 Basal IκBα expression in untreated normal mucosa and matched rectal tumours. The Y-axis shows the mean optical density of IκBα in untreated normal mucosa and matched rectal tumours in the 16 rectal cancer study patients (Subjects 1-16). SD=standard deviation

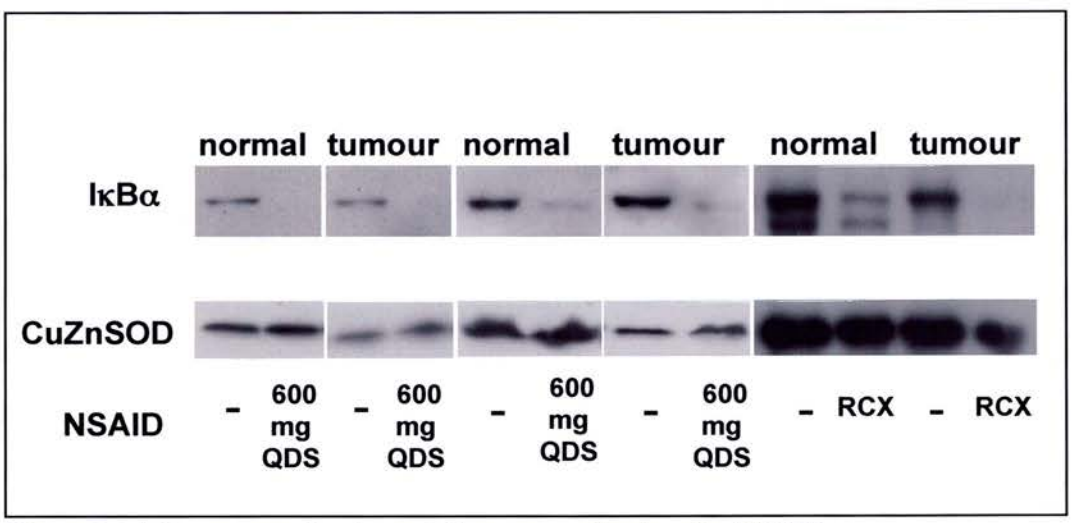

Figure 7.2 IκBα expression in rectal cancer patients after NSAIDs
Cytoplasmic protein was extracted from normal rectal mucosa and matched rectal cancer from patients treated with either aspirin or rofecoxib (RCX) for 7 days. Representative Western blots demonstrate differences in IκBα expression both in normal mucosa and cancers after NSAIDs. Copper zinc SOD used as loading control.

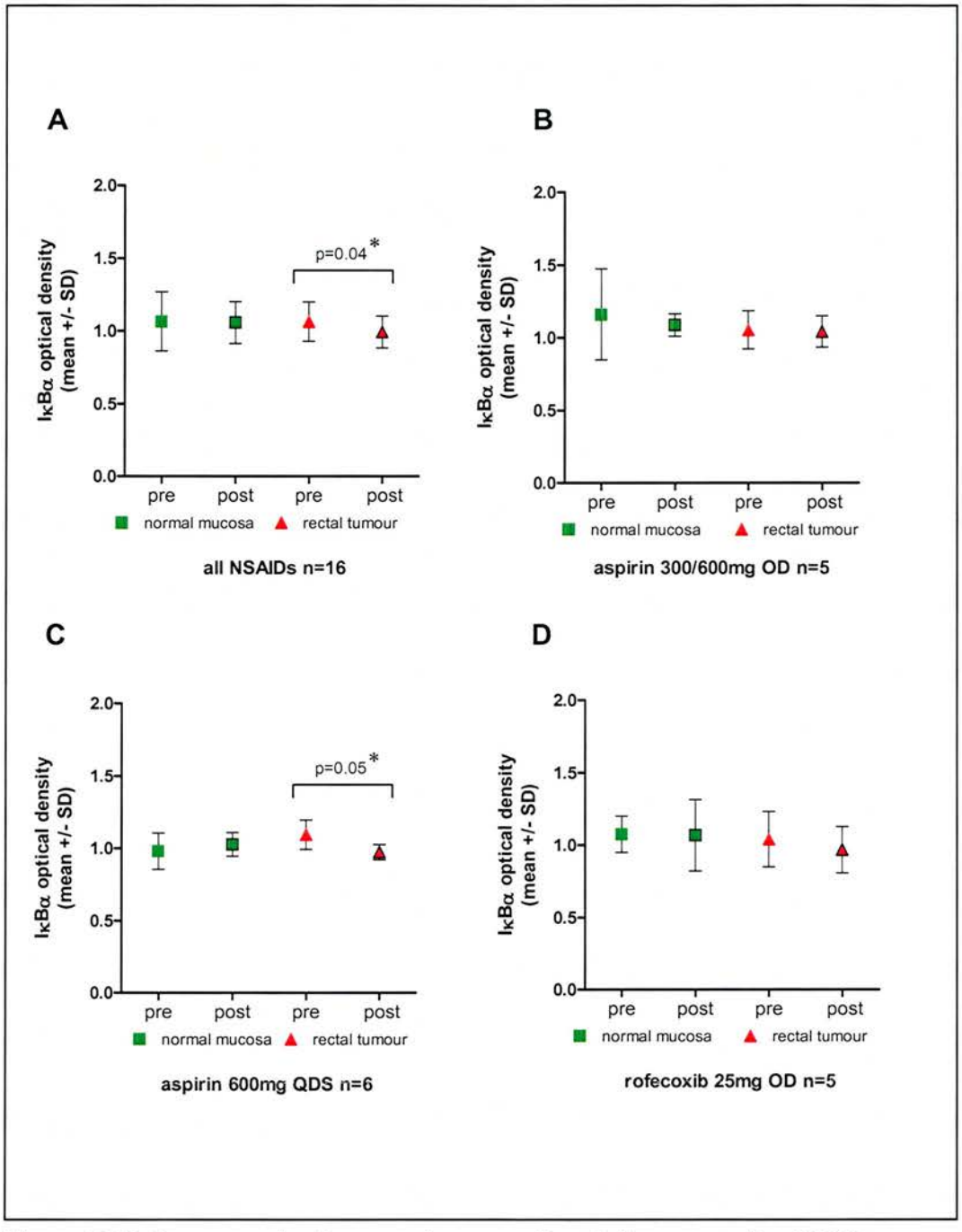

Figure 7.3 IκBα expression in normal mucosa & rectal tumours after NSAIDs
These graphs show the mean IκBα levels in the whole treatment group (**A**), the low dose aspirin group (**B**), the 600mg aspirin QDS group (**C**) and the rofecoxib group (**D**). SD=standard deviation,* Wilcoxon signed rank test

7.3.1.2 Studies in genetically predisposed individuals

7.3.1.2.1 HNPCC patients

Biopsies of normal rectal mucosa were taken from 9 patients with HNPCC before and after treatment with aspirin or rofecoxib for 1 week. Cytoplasmic IκBα protein levels were quantified by densitometry in triplicate and corrected for control protein loading and the basal IκBα levels in normal mucosa before treatment with NSAIDs are shown in Figure 7.4. Next, IκBα levels were assessed in normal mucosa before and after treatment with NSAIDs. The actual optical density values, corrected for control protein loading are shown in Table 7.2.

Table 7.2 IκBα protein expression following NSAIDs in HNPCC patients

Patient ID	NSAID Type & Dose	Normal mucosa		Difference between pre & post-NSAID IκBα	
		IκBα-pre	IκBα-post	% lower	% higher
AM	300 mg aspirin OD	1.15	0.73	36.5	-
MF		0.94	1.14	-	21.3
MB	600 mg aspirin QDS	0.89	0.96	-	7.9
RGL		1.23	1.08	12.2	-
MGL		0.93	1.19	-	28.0
BM		1.11	1.11	no change	
JSN		0.82	0.61	25.6	-
JW		1.12	0.81	27.7	-
JME	25 mg rofecoxib OD	1.09	1.01	7.3	-

The number of patients that have lower cytoplasmic IκBα expression in normal mucosa was 5 out of 9 (55.56%) and representative Western blots are shown in Figure 7.5. However, there is greater IκBα expression in normal mucosa in 3 out of 9 (33.3%) patients and no change in IκBα levels in normal in 1 patient. If a meaningful difference in IκBα expression is arbitrarily taken as ≥5% change in IκBα band intensity, then there is a decrease in IκBα in normal mucosa in 5 patients and an increase in 3 patients highlighting the variability in IκBα levels. The data presented by NSAID dose are shown in Figure 7.6, although the small numbers in individual groups preclude any consequential analysis.

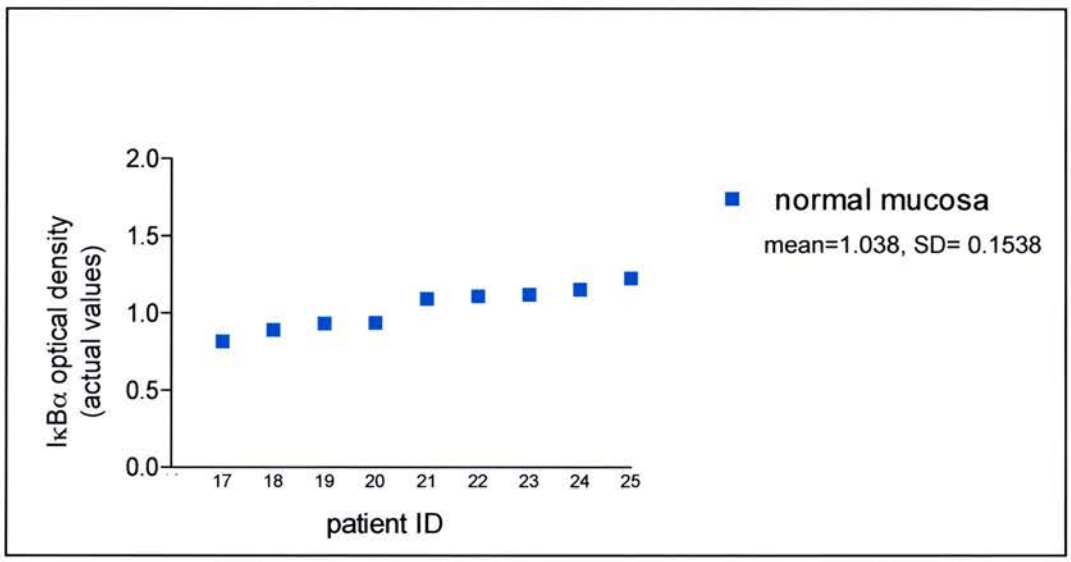

Figure 7.4 Basal IκBα expression in untreated normal mucosa HNPCC patients
The Y-axis shows the mean optical density of IκBα in untreated normal mucosa in the 9 HNPCC study patients (Subjects 17-25). SD=standard deviation

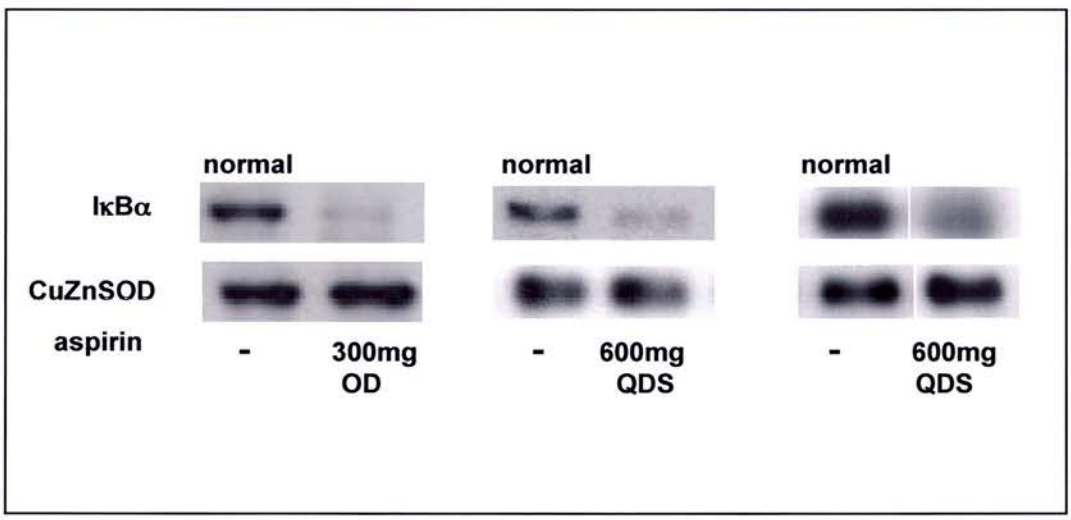

Figure 7.5 IκBα expression in HNPCC patients after NSAIDs
Cytoplasmic protein was extracted from normal rectal mucosa of HNPCC carriers treated with NSAIDs for 7 days. Representative Western blots demonstrate differences in IκBα expression in normal mucosa after NSAIDs. Copper zinc SOD used as loading control.

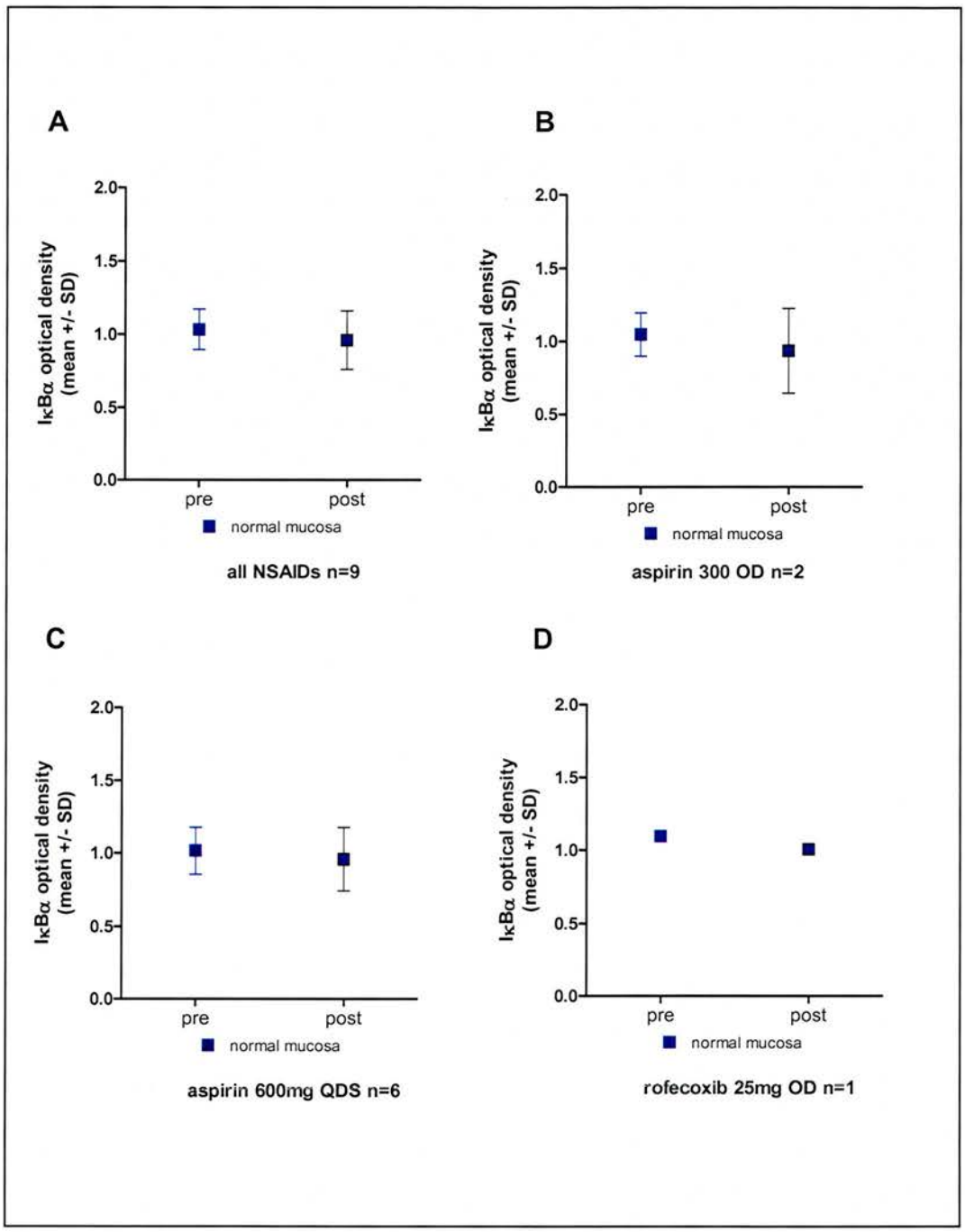

Figure 7.6 IκBα expression in normal mucosa from HNPCC patients after NSAIDs. These graphs show the mean IκBα levels in the whole treatment group (**A**), the low dose aspirin group (**B**), the 600mg aspirin QDS group (**C**) and the rofecoxib group (**D**). SD=standard deviation

7.3.1.2.2 FAP patients

Eight patients with FAP were treated with aspirin or rofecoxib, and biopsies taken from normal rectal mucosa and rectal polyps (present in 3 patients) before and after treatment. IκBα protein levels were quantified by densitometry in triplicate and corrected for control protein loading. The basal IκBα levels in normal mucosa and rectal polyps before treatment with NSAIDs are shown in Figure 7.7. IκBα levels were studied in FAP normal mucosa and rectal polyps before and after treatment with NSAIDs. The actual optical density values, corrected for control protein loading are shown in Table 7.3.

Table 7.3 IκBα levels following NSAIDs in FAP patients

Patient ID	NSAID Type & Dose	IκBα-normal		Difference between pre and post-NSAID		IκBα-polyp		Difference between pre and post-NSAID	
		pre	post	% lower	% higher	pre	post	% lower	% higher
MH	300 mg aspirin OD	1.20	1.13	5.8	-	1.04	1.04	no change	
BT	600 mg aspirin QDS	1.47	1.31	10.9	-	-	-	-	-
KW		1.18	1.14	3.4	-	1.26	1.44	-	20.3
AC	25 mg rofecoxib OD	1.11	1.35	-	21.6	-	-	-	-
DE		1.48	1.16	21.6	-	1.28	1.02	14.3	-
RG		0.96	0.97	-	1.0	-		-	-
IS		1.15	1.25	-	8.7	-		-	-
GS		0.97	1.01	-	4.1	-		-	-

The number of patients demonstrating lower cytoplasmic IκBα expression was 4 out of 8 (50%) in normal mucosa and 2 out of 3 (66.67%) in rectal polyps, and representative Western blots are shown in Figure 7.8. There is higher IκBα expression in 4 (50%) normal mucosa specimens and in 1 (12.5%) rectal polyp sample. IκBα expression in the different NSAID treatment groups is shown in Figure 7.9. If a 5% change in IκBα expression is taken as a significant change, then there is lower IκBα in normal mucosa in 3 patients (37.5%), but also higher IκBα band intensity in 3 patients (37.5%). In the FAP polyp group, there is a decrease in IκBα levels in 1 patient and an increase in 1 patient. Hence, it is clear that the extent of IκBα variability needs to be elucidated further.

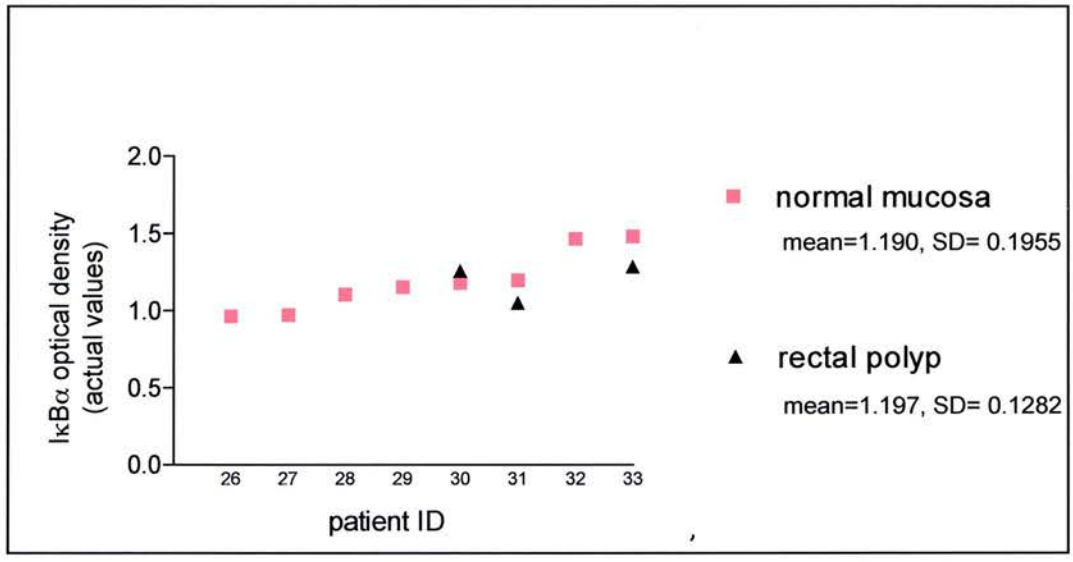

Figure 7.7 Basal IκBα expression in untreated normal mucosa and matched rectal polyps from FAP patients The Y-axis shows the mean optical density of IκBα in untreated normal mucosa of 8 FAP patients and matched rectal polyps in 3 patients (Subjects 26-33). SD=standard deviation

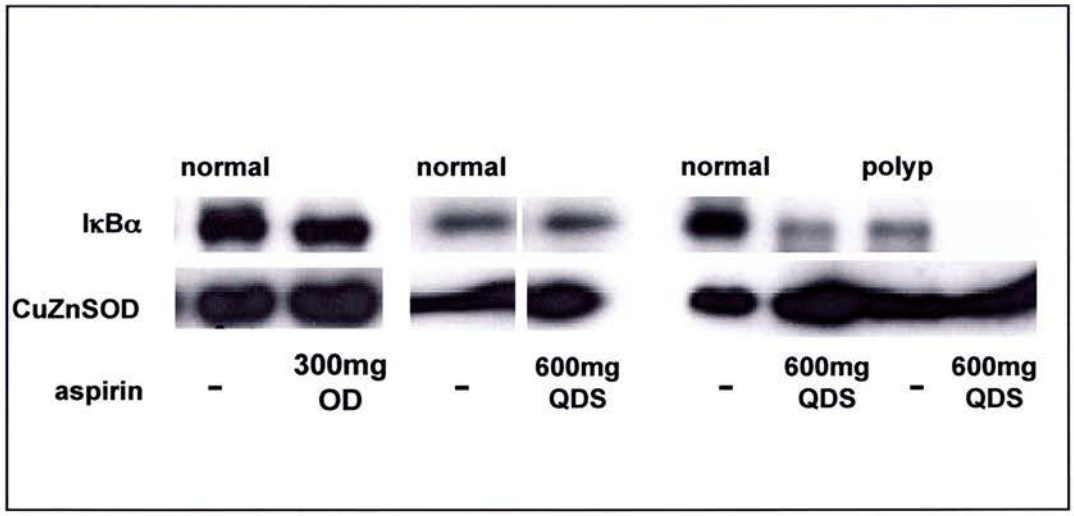

Figure 7.8 IκBα expression in FAP patients after NSAIDs
Representative Western blots show that IκBα expression in normal mucosa and polyp tissue in FAP patients. Copper zinc SOD was used as loading control.

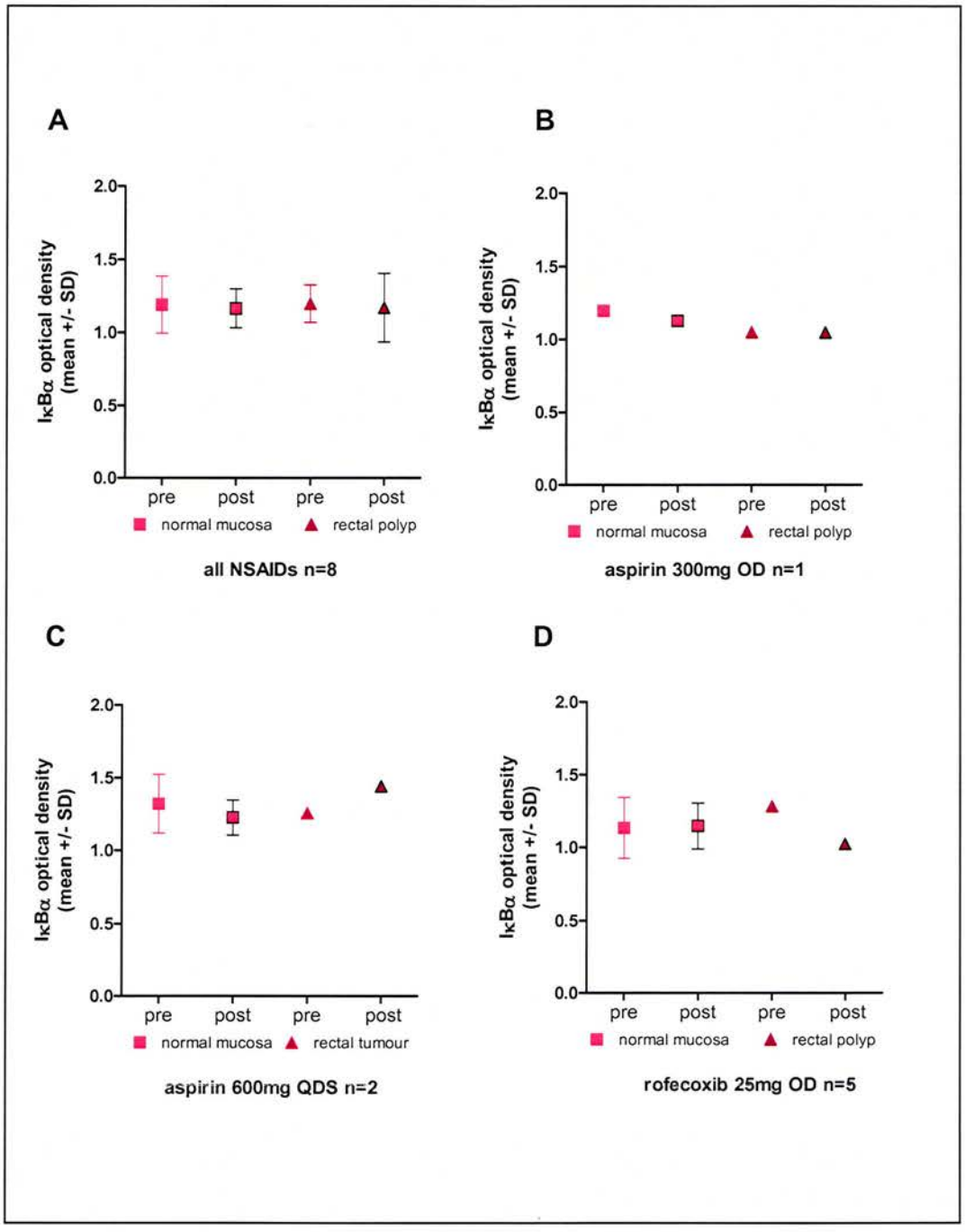

Figure 7.9 IκBα expression in normal mucosa & rectal polyp in FAP after NSAIDs. These graphs show the mean IκBα levels in the whole treatment group (**A**), the low dose aspirin group (**B**), the 600mg aspirin QDS group (**C**) and the rofecoxib group (**D**). SD=standard deviation

7.3.2 Apoptosis in rectal cancer after NSAIDs

Apoptosis was assessed by counting apoptotic bodies in H&E stained sections of biopsies from rectal cancer patients before and after treatment with NSAIDs. The apoptotic counts were calculated using a running mean method (described in Section 2.5.3) and are shown in Table 7.4.

Table 7.4 Apoptotic counts following NSAIDs in rectal cancer patients

Patient ID	NSAID Type & Dose	Apoptosis-normal		Difference between pre and post-NSAID	Apoptosis-tumour		Difference between pre and post-NSAID
		Pre	Post		Pre	Post	
AB	300 mg aspirin OD	0	0	no change	0.20	0.40	higher
SF		0	0	no change	1.77	0.63	lower
HG	600 mg aspirin OD	0.07	0	lower	0.20	0.27	higher
JS		0	0	no change	0.03	0.83	higher
MC		0	0	no change	3.00	1.13	lower
DB	600 mg aspirin QDS	0.50	2.50	higher	1.90	1.95	higher
JC		0	0	no change	0.50	0.65	higher
JM		-	-	-	0.73	0.60	lower
JL		0	0.50	higher	0.60	1.00	higher
JP		0	0	no change	0.50	0.95	higher
ES		0	0	no change	0	0.60	higher
JK	25 mg rofecoxib OD	0	0	no change	0	0.35	higher
KW		0	0	no change	0	0.25	higher
DW		0	0.33	higher	0.50	0.60	higher
AW		0	0	no change	0	1.70	higher
GW		0	0	no change	3.37	4.03	higher

It was essential to have a validated measure of apoptosis performed by a pathologist experienced in the technique, and hence the apoptotic counts were kindly done by Dr Angus McGregor who was blinded to the treatment regimens.

There is variability in the basal levels of apoptosis in both normal mucosa and rectal cancers, but the basal level of apoptosis of rectal cancers as a group was greater than normal mucosa as a group ($p= 0.007$, Wilcoxon signed rank test) (Figure 7.10A). In terms of overall response, there were greater apoptotic counts in rectal tumour biopsies of 13 patients (81.3%) and in normal mucosa of 3 patients (18.7%) (Figure 7.10B). The apoptotic response was marginally greater in rectal tumours compared to normal rectal mucosa when examining the whole group of NSAIDs (Figure 7.11A). There is tentative evidence for a dose-dependent effect since there was no increase in apoptotic counts compared to pre-treatment levels in either normal rectal mucosa or in rectal tumours following treatment with low-dose aspirin, 300 or 600 mg once daily (Figure 7.11B). However, the apoptotic counts were greater following treatment with the 2.4g aspirin dose and with rofecoxib in both normal mucosa and rectal tumours (Figure 7.11 C&D). These results suggest that both COX-2 selective and non-specific NSAIDs may induce apoptosis in rectal tumours of the majority of NSAID treated patients.

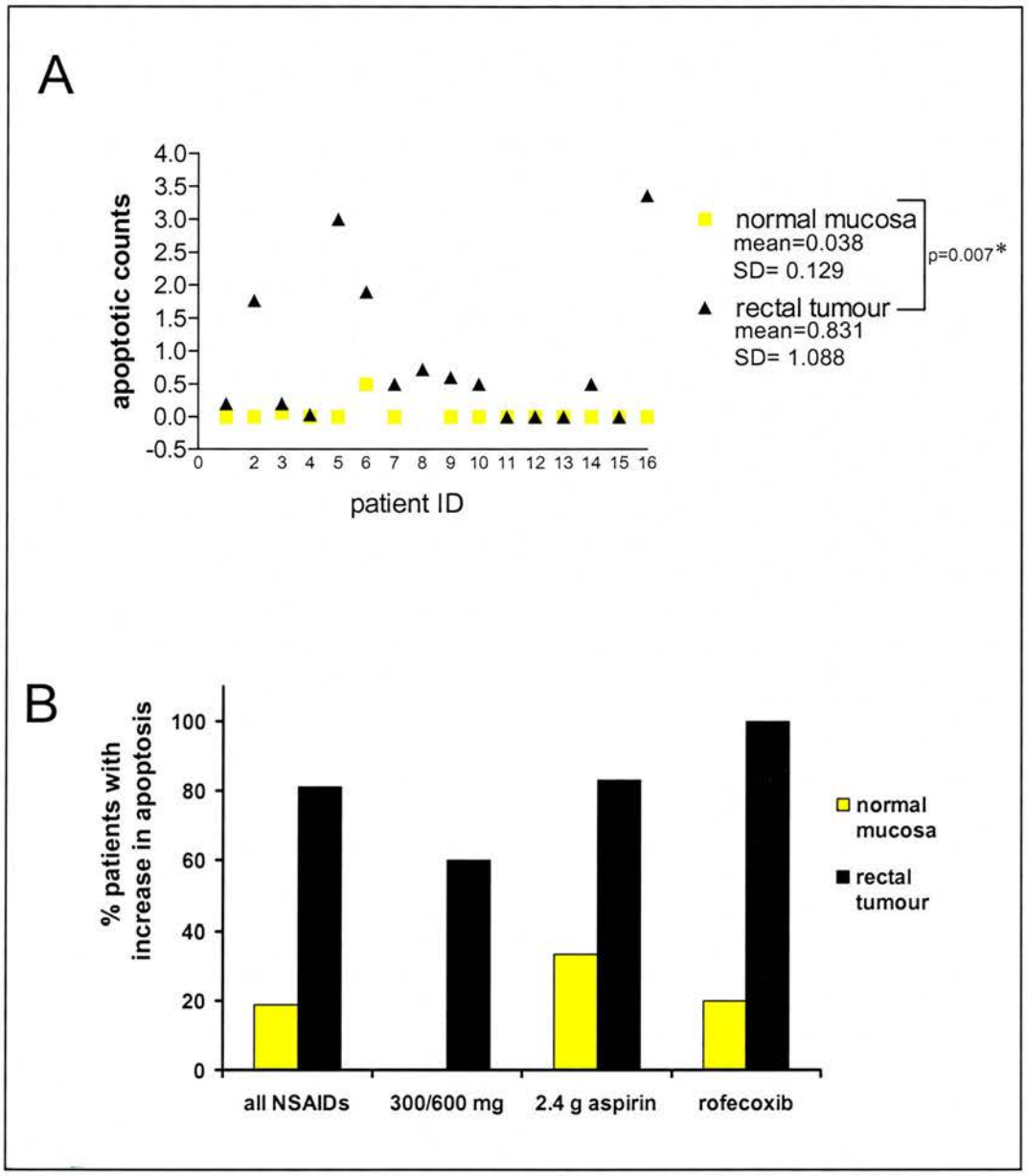

Figure 7.10 Apoptosis in rectal cancer patients after NSAIDs
Basal pre-treatment apoptotic counts in normal mucosa and matched rectal cancers (**A**). There was increased apoptosis in tumour biopsies of 13 patients and in normal mucosa of 3 patients (**B**).SD=standard deviation,* Wilcoxon signed rank test

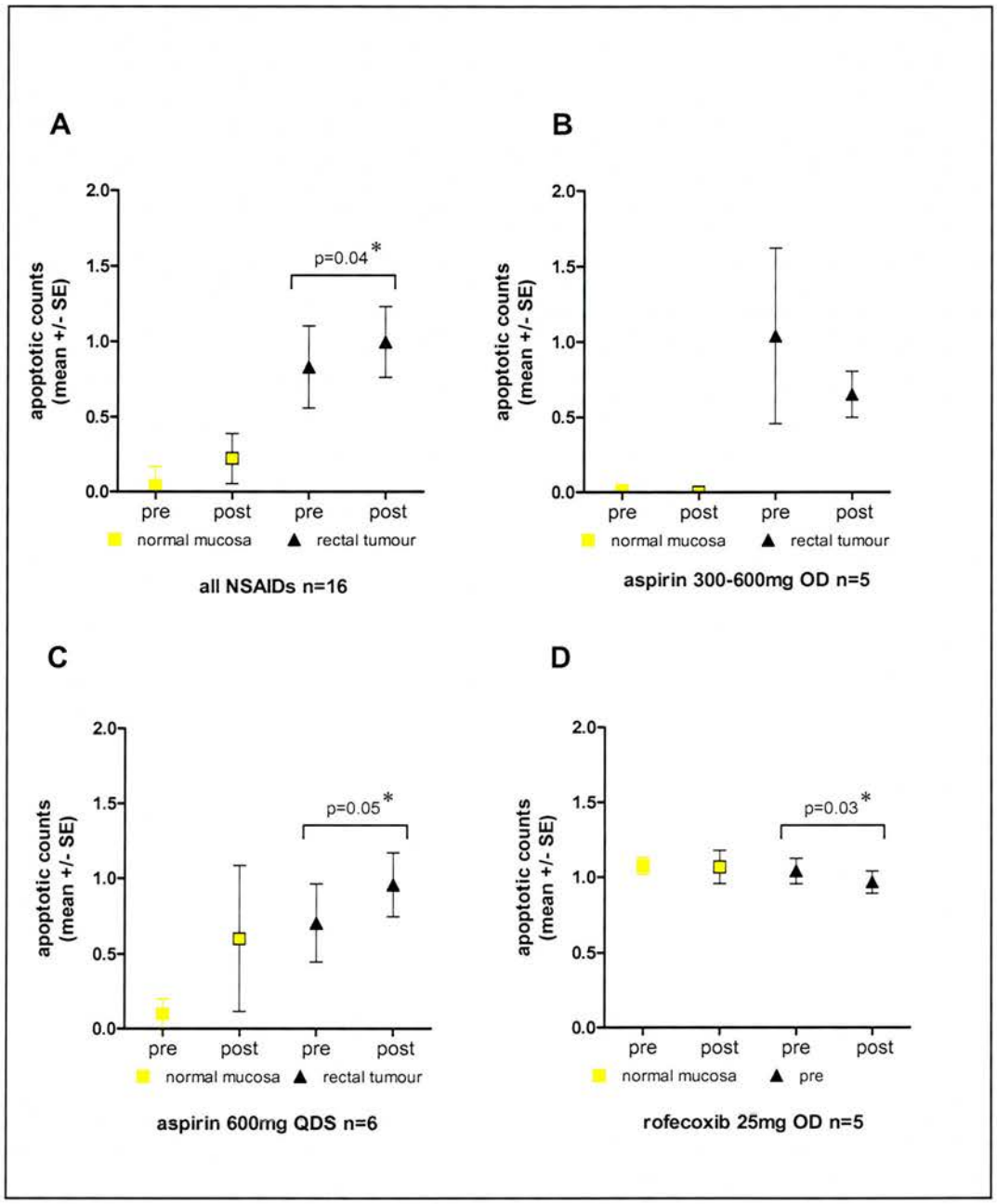

Figure 7.11 Apoptosis in normal mucosa and rectal tumours after NSAIDs
These graphs show the mean apoptotic counts (+/- SE) in the whole rectal cancer study group (**A**), the low dose aspirin group (**B**), the 600mg aspirin QDS group (**C**) and the rofecoxib group (**D**). SE=standard error ,* Wilcoxon signed rank test

7.3.3 Serum salicylate

Epidemiological studies have focussed on populations using NSAIDs for their analgesic effects and there are no definitive data on the dose required for a chemopreventive effect. It is also important to investigate the relevance of the aspirin concentrations to those used in the in *vitro* experiments described in earlier chapters. Hence, venous blood was drawn from subjects who were administered aspirin 300mg aspirin OD (n=2), 600mg aspirin OD (n=4) and 600mg aspirin QDS (n=8) for 7 days. Serum salicylate levels were measured by a standard colorimetric assay of Trinder by the Department of Clinical Biochemistry (Western General Hospital, Edinburgh). Measurements of serum salicylate levels in 14 study subjects ranged from 0.05 to 1.13 mM (mean 0.37 mM, SE +/- 0.13 mM) and Figure 7.12 shows that the dose of aspirin ingested correlates, to some extent, with the serum salicylate levels (r=0.69, correlation coefficient).

7.3.4 Potential side-effects of study

There were 2 patients with rectal cancers who felt that there was an increase in bleeding per rectum after the second set of biopsies, following NSAID ingestion, and were admitted for overnight observation. It is difficult to know whether this was due to aspirin or simply an exacerbation of pre-existing symptoms but no further problems were encountered.

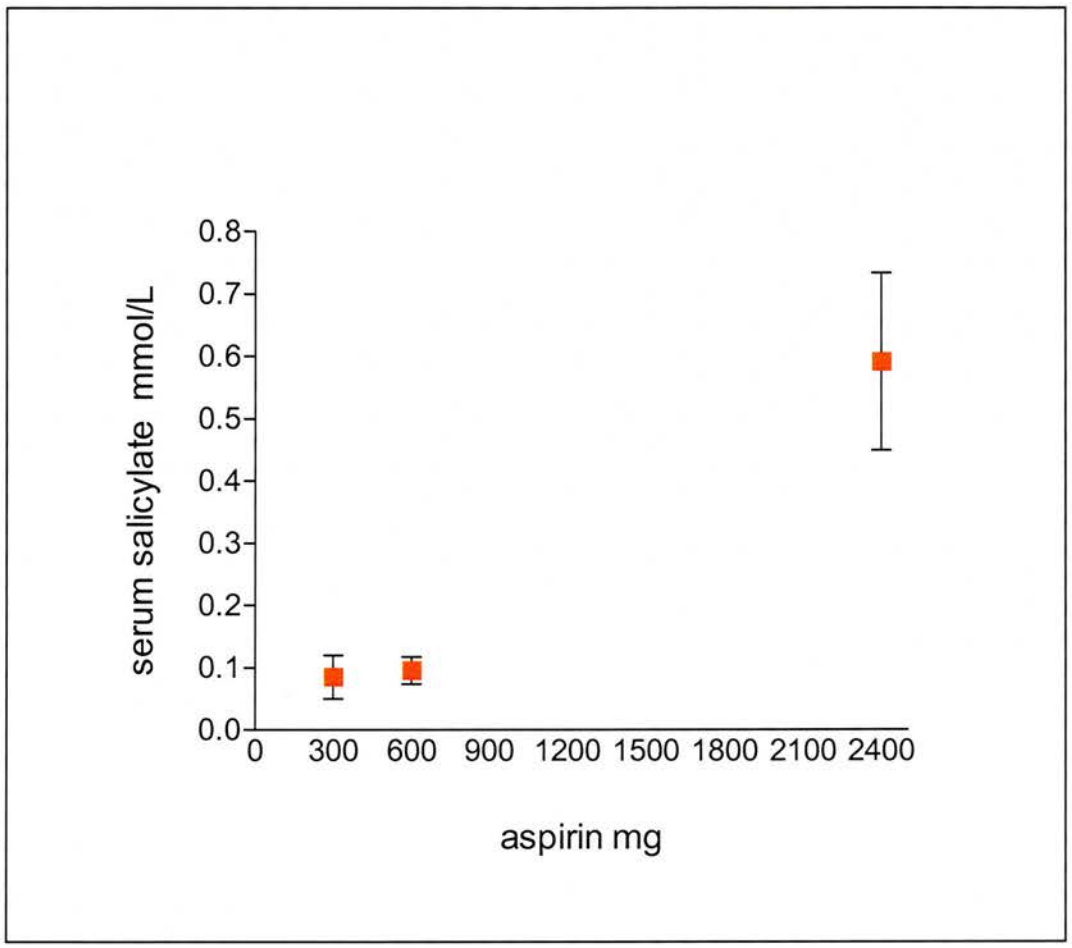

Figure 7.12 Serum salicylate levels in patients after aspirin
The Y-axis shows the mean (+/- SE) serum salicylate levels in mmol/L. The X-axis shows the doses of aspirin (mg/day) in patients ingesting 300mg aspirin once daily (n=2), 600mg aspirin once daily (n=4) and 600mg aspirin four times daily (n=8). SE=standard error

7.4 Discussion

Despite epidemiological evidence showing that NSAIDs protect against colorectal cancer with a 40-50% reduction in relative risk (Thun *et al.*, 1993), it would be unwise at present to recommend their widespread use for chemoprevention purposes. NSAIDs are associated with adverse gastrointestinal effects including bleeding and perforated peptic ulcers (Bidaut-Russell and Gabriel, 2001). The lifetime risk of colorectal cancer is 1 in 20 and the risk of a fatal complication has been estimated at 1 in every 2000 NSAID prescription over 2 months duration (Tramer *et al.*, 2000). Selective COX-2 inhibitors have been designed to reduce gastrointestinal morbidity and although associated with fewer ulcers, it is unclear whether such small endoscopically-defined ulcers predict complications (Wright, 2002). Interestingly, focus on COX-2 related drug development has revealed that COX-2 is integral to gastrointestinal mucosal defence (Wallace and Devchand, 2005). In addition, since development of COX-2 inhibitors, there is a risk that more people in general are commenced NSAIDs thereby increasing the overall associated risks (Girvin *et al.*, 2004). The COX-2 selective inhibitor rofecoxib was withdrawn due to increased risk of sudden cardiac events and deaths that were observed in a polyp prevention trial (Bresalier *et al.*, 2005). Indeed, a recent study showed that COX-2 inhibitors were not any safer than conventional NSAIDs (Hippisley-Cox *et al.*, 2005). Hence, it is evident that the COX-2 selective NSAIDs will not be ideal chemopreventive agents. Given the considerable protective effect of NSAIDs in clinical studies, there is compelling rationale to identify the molecular mechanism of action in order to inform new and safer drug design.

The work outlined in the previous chapters has identified that the NFκB signalling pathway is central to aspirin-induced apoptosis in colorectal cancer cells. However, the dynamics of this pathway and the contribution that specific NFκB complexes make to a physiological response are relatively ill defined *in vivo* (Barkett and Gilmore, 1999). The aim of this work was to investigate whether the *in vitro* findings translate into modulation of the NFκB signalling pathway by NSAIDs in patients, and to determine whether pharmacologically relevant doses could induce a detectable effect in pilot clinical studies using pre-treatment biopsies within patients as controls. It is well known that NFκB plays a role in regulation of cell proliferation and death in several cell types including epithelial cells in the colon (Inan *et al.*, 2000). In mouse colon, expression of the p65 NFκB subunit is greater in proliferating cells at the crypt base compared to mature cells at the crypt surface (Inan *et al.*, 2000). The decreased NFκB activity observed at the crypt surface is consistent with the observation that the inhibitor IκBβ is highly expressed in mature surface colonic epithelial cells (Wu *et al.*, 1999). Decreased NFκB activity at the crypt surface may contribute to dampened NFκB responses developed by intestinal epithelial cells to preserve homeostasis in an otherwise hostile colonic environment (Jobin and Sartor, 2000). The dynamic nature of NFκB pathway constituent expression, during epithelial cell maturation and progression up the colonic crypt, indicates that NFκB may participate in regulating epithelial cell turnover (Inan *et al.*, 2000). Increased constitutive activity of NFκB has been reported in colorectal cancer especially poorly differentiated and mucinous tumours (Lind *et al.*, 2001; Evertsson and Sun, 2002). Increased NFκB expression may also contribute to colorectal cancer angiogenesis through increased VEGF expression (Yu *et al.*, 2004). Taken together these data suggest that NFκB may play an important role in the pathogenesis of colorectal carcinoma.

These pilot clinical studies are the first to examine aspirin effects *in vivo* and in particular focussing on NFκB. However, this analysis cannot assess the dynamic state of IκBα, as it is based on static measurement of IκBα protein levels by Western blotting. The results show that IκBα protein expression can be detected in normal mucosa and rectal cancer biopsies, but that there was considerable variability both in basal and post-treatment IκBα levels. There are several potential sources of variability that may be related to biopsy sampling, such as the time of day when biopsied or the effect of the sigmoidoscopy and biopsy itself, or may be related to the dose of NSAID. Indeed, the changes in IκBα expression may be due to inherent variability of the protein and unrelated to treatment with NSAIDs. Therefore, it will be important to further define intra-patient variability in terms of IκBα expression. Hence, IκBα expression will be determined in separate biopsies of untreated normal mucosa and rectal cancers taken at the same time-point from the same patient. Basal IκBα expression will also be studied in untreated normal mucosa and rectal tumours taken at the different study time-points (0 and 7days). Although the *in vitro* experiments (Chapter 3) showed that NSAIDs degrade IκBα, any decrease in IκBα protein expression may be due to degradation of the protein or decreased protein transcription, or may be due to variability in experimental detection. Subsequent work has suggested that IκBα is being degraded, since there is an increase in IκBα phosphorylation *in vivo* after NSAIDs and phosphorylation is known to precede IκBα degradation (Din *et al.*, 2005).

There were no significant differences between basal expression of IκBα protein in matched normal and tumour specimens that would indicate altered basal levels of IκBα in tumours. However, due to small numbers, subtle differences cannot be excluded. The

differences in IκBα levels following NSAID treatment may be real but given the degree of variability this cannot be confirmed within the experimental conditions used here. While the IκBα expression changes induced by NSAIDs could only be small, there is evidence in *Drosophila* that small differences in nuclear concentrations of NFκB complexes can result in profound physiological differences (Govind, 1999).

NFκB signalling can result in regulation of pro or anti-apoptotic genes depending on the stimulus, cell type and the composition of the activated complex (Barkett and Gilmore, 1999). Apoptosis not only regulates cell number and type, but also eliminates damaged cells and those with DNA aberrations, thereby preventing proliferation of malignant clones (Pritchard and Watson, 1996). Interestingly, there is a lower incidence of spontaneous apoptosis in the colon compared with the small intestine, which may account for higher incidence of colon cancers as a result of cells harbouring mutations not being eliminated (Potten, 1992). Indeed progressive inhibition of apoptosis has been observed in the transition from normal colonic epithelium to adenomas and carcinomas (Bedi *et al.*, 1995). In these studies, apoptosis has only been studied in the rectal cancer group thus far. Clearly the data generated by the apoptotic counts is subject to the same issues related to variability. The data show greater apoptotic counts in tumour biopsies of 13 patients (81.3%) and in normal mucosa of 3 patients (18.7%) after NSAIDs. In normal colonic epithelium, spontaneous apoptosis is not as common as in the small intestine and apoptotic counts tend to be less than 1.0. Counting of apoptotic bodies is the best-validated method for quantification, but the half-life of these fragments varies according to the apoptosis-inducing agent and determines the number of fragments seen at a single time point (Pritchard and Watson, 1996). Apoptosis is rapid and often completed within

4-9 hours (Pritchard and Watson, 1996). The post-treatment biopsies were all taken within 16 hours of the final NSAID dose. It may be that NSAIDs induce an initial burst in apoptosis, followed by a persistent but lower level increase in apoptotic turnover. The timing of aspirin-induced apoptosis in colorectal epithelium is being explored in new clinical studies in the host laboratory. There is evidence that low levels of apoptosis translate into significant tumour regression over time in cell kinetics studies (Pritchard and Watson, 1996). These results are consistent with other *in vivo* work showing increased apoptosis in colonic epithelium from patients with NSAID-induced colitis (Lee, 1993) and in adenomas of sulindac treated FAP patients (Keller *et al.*, 1999).

It is also important to note that the biopsies of both normal mucosa and rectal tumour, although predominately epithelial, would also include stromal cell populations. Hence, any effect of NSAIDs on NFκB signalling may be even more pronounced if examining an isolated epithelial colonic cell population. Alternatively, aspirin may affect numerous populations including stromal, epithelial, and colonic crypt stem cells. Investigation of the effects of aspirin on these individual cell types using microdissection may shed further light as to the relative contribution of both direct epithelial and indirect paracrine-type effects on NFκB signalling.

This *in vivo* research has provided some interesting insights into study of the effects of aspirin on NFκB signalling. It has highlighted the difficulties of translating laboratory research to 'proof of principle' pilot clinical studies and the importance of clinical trial design when investigating molecular end-points. The results show that IκBα protein expression can be detected in both normal mucosa and rectal tumours of rectal cancer and

genetically-predisposed patients but that there is considerable degree of variability, which requires further investigation. The variability may be due to confounding physiological factors, sampling and experimental variation or indeed may be real. Studies investigating the variability in IκBα in normal mucosa and rectal cancers of untreated patients are underway following ethical approval. This work has also informed new clinical studies which include shorter time-points to further delineate the kinetics of mechanism of action *in vivo* and recruitment has commenced following ethical approval. It is also necessary to investigate the effects of aspirin on NFκB activity and effects on relevant downstream genes. This work is also ongoing. It will be also be important to determine whether NFκB modulation can be used as a surrogate endpoint in clinical chemoprevention trials. Chemoprevention trials are problematic due to the duration, the numbers of patients required to show clinical benefit and the cost involved. Hence, identification of a molecular surrogate marker of response would be a major advance.

Chapter 8

Mutation analysis of *Rel A* and *IkBa* genes in colorectal cancer tissue

8.1 Introduction

The functions of the NFκB signalling pathway are intimately involved in carcinogenesis, and altered regulation of NFκB has been reported in colorectal cancer. The hypothesis explored in this chapter is that aberrant NFκB signalling in colorectal cancer may be due to mutations in genes encoding key components of the pathway. To date there have been no studies examining the mutation status of genes encoding proteins involved in NFκB signalling in colorectal cancer. In this chapter, *Rel A* (synonymous with *p65)* and *IkBa* were investigated as strong candidates due to the central role played by the respective proteins, p65 and IκBα, in aspirin-induced effects on NFκB signalling. Mutations in either gene may be responsible for the variability in NFκB response observed in rectal cancer patients (Chapter 7) and the cell-type specificity of the response, and providing rationale for investigation of genetic aberrations of *Rel A* and *IkBa*.

8.1.1 *Rel A* genomic structure and function

The human *Rel A* gene comprises 10 exons spanning about 8.1 kb (Deloukas and van Loon, 1993). *Rel A* is located on chromosome 11q12-q13 (Deloukas *et al.*, 1994). The structure and important functional domains and amino acids are represented schematically in Figure 8.1. Exon 1 contains the 5' untranslated region of mRNA and codes the first 12 amino acids of p65. Exons 2-7, approximately 300 amino acids, make up the Rel homology domain (RHD) of which the first 200 amino acids are involved in specific DNA binding and last 100 amino acids in dimerisation. The nuclear localisation signal (NLS) consisting of amino acids 301-304 (KRKR) is located in exon 8. The *Rel A* gene encodes the p65 subunit of NFκB which is responsible for transcriptional activity. There are two transactivation (TA) domains that are present in exon 10, TA1 comprises the last 30 amino acids and TA2 is made up of the 90 amino acids immediately adjacent to TA1. There is also a putative nuclear export sequence, LSEALLQLQF, in the second transactivation domain of exon 10 (Harhaj and Sun, 1999).

The crystal structure of the NFκB-IκBα complex has been determined (Huxford *et al.*, 1998; Jacobs and Harrison, 1998). There are several regions of contact between p65 and IκBα; ankyrin repeats 1 and 2 of IκBα contact the c-terminal extension of p65 including the NLS, and repeats 5 and 6 contact the dimerisation domain of p65 (Jacobs and Harrison, 1998). The nuclear localisation sequence of p65 is sequestered by the first two ankyrin repeats of IκBα. This is the mechanism for cytoplasmic retention of p65 by IκBα. Indeed, mutations involving the NLS of p65 may interfere with the ability of IκBα to mask the NLS, rendering NFκB continuously activated.

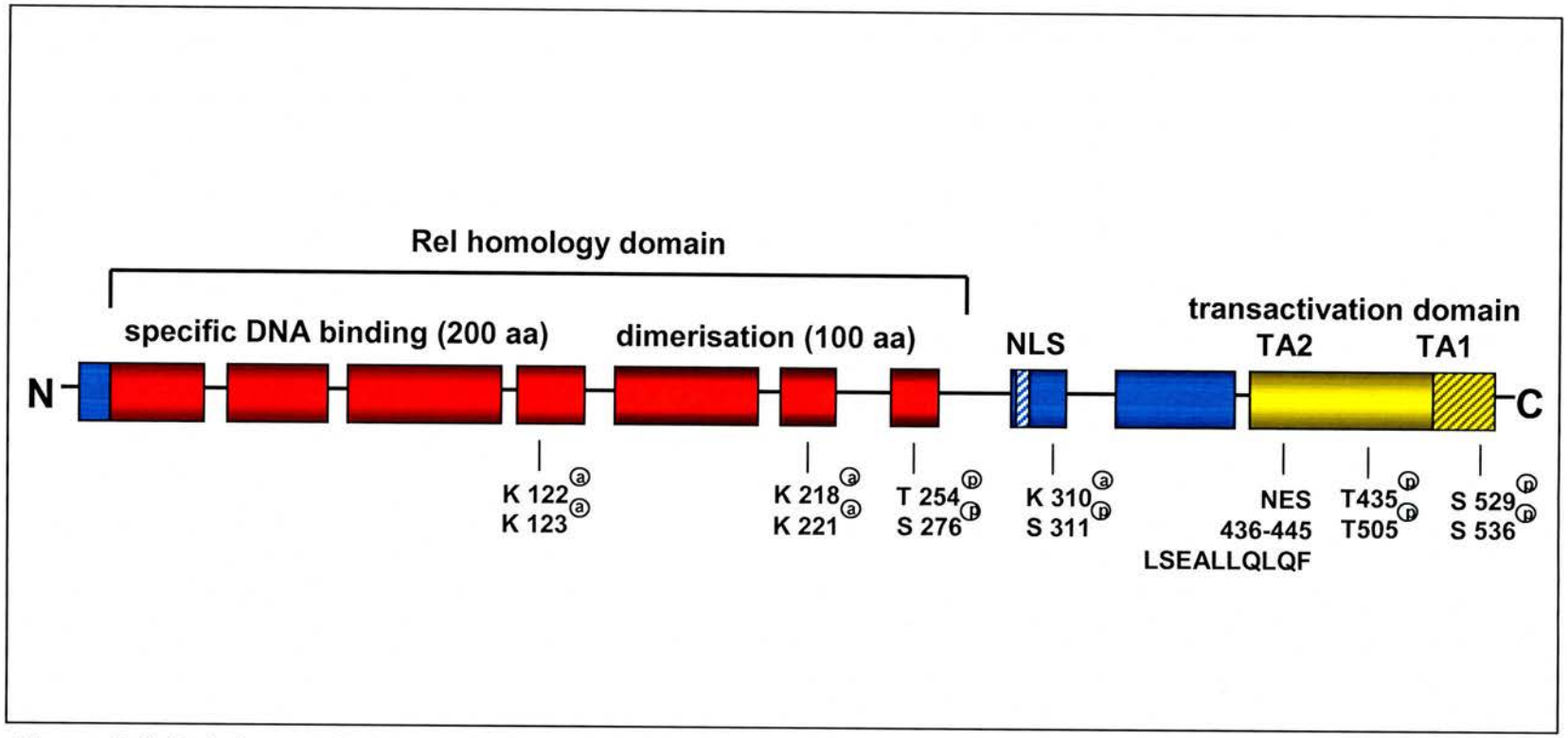

Figure 8.1 *Rel A* gene Rel homology domain (RHD) made up of exons 2-7(~300 amino acids) of which first 200 amino acids are involved in specific DNA binding and last 100 amino acids in dimerisation. Nuclear localisation signal (NLS) from amino acids 301-304 (KRKR) in exon 8. Two transactivation (TA) domains in exon 10, TA1 is the last 30 amino acids and TA2 comprise the 90 amino acids immediately adjacent to TA1. Potential nuclear export sequence (NES), LSEALLQLQF, in TA2. Acetylation and phosphorylation sites marked as ⓐ and ⓟ.

Although, the classical pathway for NFκB activation is one where IκBα undergoes degradation permitting NFκB nuclear translocation, there are other mechanisms that contribute to NFκB activation. Increasingly, post-translational modifications have been shown to play an important role in activation of p65 (Campbell and Perkins, 2004). A number of stimuli are known to phosphorylate p65 at different serine residues, such as Pin-1 at T254 (Ryo *et al.*, 2003), PKA at S276 (Zhong *et al.*, 1997), PKCz at S311 (Duran *et al.*, 2003), casein kinase II at S529 (Wang *et al.*, 2000) and NIK/IKK at S536 (Jiang *et al.*, 2003). Phosphorylation at these sites increases p65 transcriptional activity, in contrast to phosphorylation at T435 by protein phosphatase 4 and at T505 by ARF which results in inhibition (Rocha *et al.*, 2003; Yeh *et al.*, 2004). More recently, acetylation sites have been identified at lysine residues. Acetylation by p300 and PCAF at K122 and K123 reduces p65 binding to DNA (Kiernan *et al.*, 2003), whereas acetylation by p300 and CBP acetyltransferases at K218, K221 and K310 increase p65 transcriptional activation (Chen *et al.*, 2002). Hence, a polymorphism or mutation at any one of these sites may affect target gene transcriptional specificity of NFκB, which is clearly regulated at several levels.

8.1.2 *IkBa* genomic structure and function

The *IkBa* gene maps to chromosome 14q and consists of 6 exons spanning approximately 3.5kb, which are schematically represented in Figure 8.2. Exon 1 encodes the signal receiving domain (SRD) which does not physically participate in NFκB-IκBα complex formation (Hatada *et al.*, 1992; Jaffray *et al.*, 1995; Sun *et al.*, 1996). However, exon 1 contains key serine residues, S32 and S36, which are important phosphorylation sites and

lysine residues upstream, K21 and K22, which undergo ubiquitination that targets the protein for degradation by the 26S proteosome. Approximately the last 10 amino acids of exon 1 and exons 2-5 comprise the ankyrin repeat domain (ARD). There are 6 ankyrin repeats, about 33 amino acids each, which form 5 finger-like projections that interact with the dimerisation domain of p65 (Huxford *et al.*, 1998). The NLS of p65 is sequestered by the first 2 ankyrin repeats and repeats 4-6 contact the RHD of p65 (Jacobs and Harrison, 1998). Hence, mutations in *IkBa* may affect both its degradation by effects on phosphorylation and ubiquitination sites, and its ability to bind to p65, by direct effects on the p65 interacting domain or by indirectly affecting protein structure and NFκB-IκBα complex formation, which may disrupt NFκB regulation.

There are chromosomal regions and genetic loci that undergo loss of heterozygosity in colorectal cancer including *APC* at 5q, *p53* at 17p and *DCC* at 18q. Interestingly, LOH has been reported both at chromosome 11q and chromosome 14q in colorectal cancer (Tomlinson and Bodmer, 1996; Weber *et al.*, 1999; Lee *et al.*, 2000; Thorstensen *et al.*, 2001). Moreover, chromosome 14 monosomy has been reported in both primary and metastatic colorectal cancer (Thorstensen *et al.*, 2001). Indeed, loss of the entire chromosome or a large part may confer a selective growth advantage due to loss of several tumour suppressor genes simultaneously. More recently, genome-wide linkage analysis of colorectal cancer families in Sweden identified regions of interest in chromosomes 11 and 14, suggesting these may harbour novel predisposition genes (Djureinovic *et al.*, 2005).

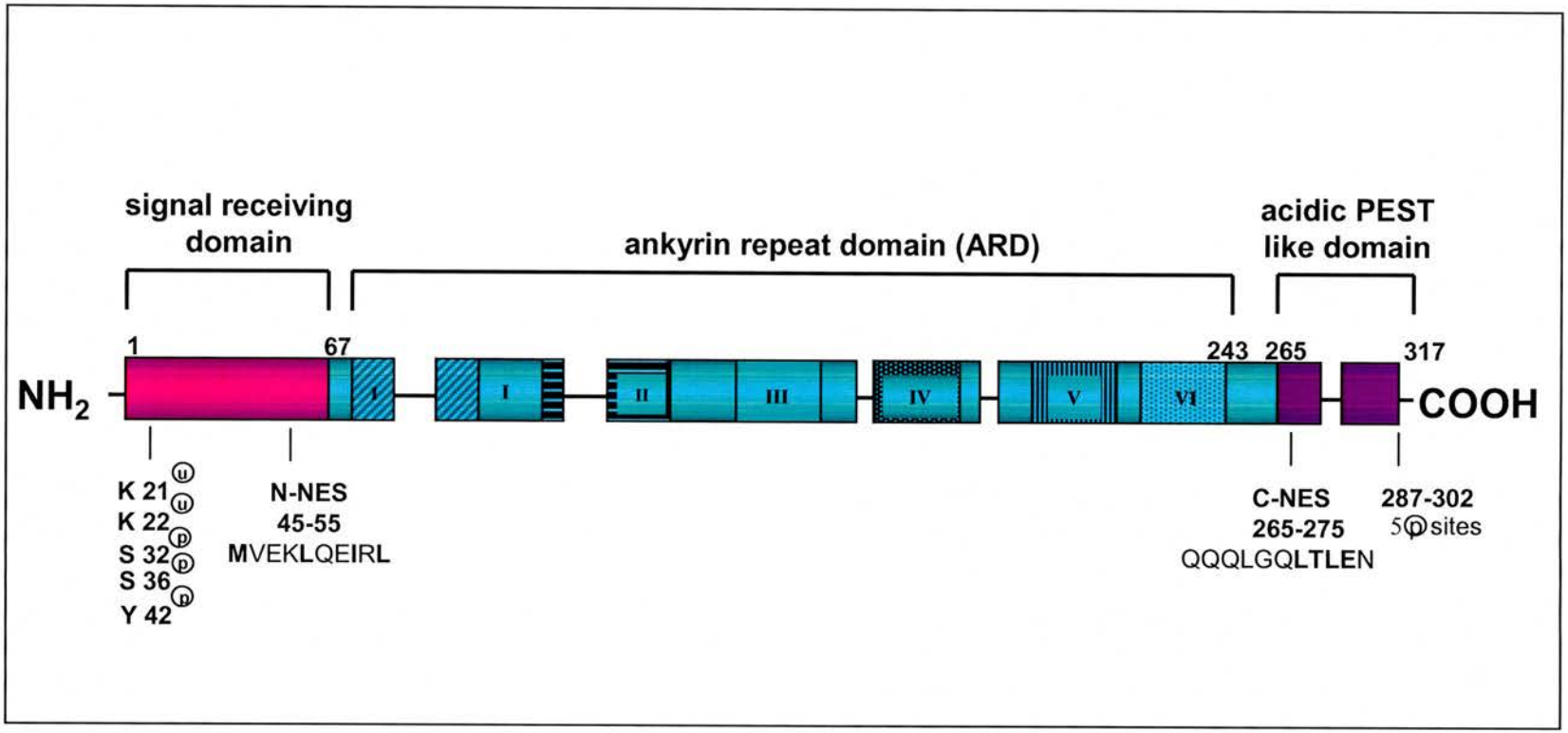

Figure 8.2 *IkBa* gene. Signal receiving domain (SRD) in exon 1. Phosphorylation and ubiquitination sites marked as ⓟ and ⓤ Ankyrin repeat domain (ARD) consists of last 10 amino acids of exon 1 and exons 2-5. Six ankyrin repeats (I-VI) ~33 amino acids each, which interact with dimerisation domain of p65. NLS of p65 is sequestered by the first 2 ankyrin repeats and repeats 4-6 contact the RHD of p65.Two nuclear export sequences (NES) in N and C termini. 5 CKII phosphorylation sites in within amino acids 287-302 .

8.1.3 NFκB signalling and carcinogenesis

NFκB regulates numerous pathways involved in oncogenesis including the cell cycle, cell differentiation, cell migration and control of apoptotic signalling. NFκB may promote carcinogenesis through its modulation of genes governing the balance between cell proliferation and cell death. The proto-oncogene *c-myc* and cell cycle protein cyclin D1 are targets of the APC pathway (He *et al.*, 1998; Shtutman *et al.*, 1999). Mutations in *Wnt* signalling are common in colorectal cancer resulting in increased Lef-1/Tcf-driven expression of both c-myc and cyclin D1. The *c-myc* promoter contains an NFκB binding site and gene expression is stimulated by NFκB, leading to aberrant cell proliferation (Pahl, 1999). *c-myc* has also been shown to induce a truncated form of Rel A with which it forms a complex (Chapman *et al.*, 2002). NFκB also induces expression of cyclin D1, through promoter NFκB binding sites and so indirectly regulates G1 to S phase cell cycle progression (Guttridge *et al.*, 1999). Interactions between NFκB and other cell cycle regulators, including *Rb, p53* and *p21* genes, may also influence cell proliferation and survival (Hinata *et al.*, 2003).

In addition, NFκB regulates genes encoding growth factors including bone morphogenetic protein-2 (Feng *et al.*, 2003) and platelet derived growth factor (Khachigian *et al.*, 1995). These genes are involved in clonal expansion and perhaps tumour initiation. Several cytokines are regulated by NFκB including interleukins, RANTES (Moriuchi *et al.*, 1997) and TNFα (Collart *et al.*, 1990). These act as tumour cell growth factors, and may result in continual autocrine or paracrine stimulation of cells that are already 'primed' through mutation and clonal selection. In addition to regulating

genes involved in cell proliferation, NFκB signalling may also contribute to growth of new blood vessels due to regulation of genes involved in angiogenesis such as *VEGF* (Chilov *et al.*, 1997), and to this end increased NFκB expression has been shown to correlate with increased VEGF expression (Yu *et al.*, 2004).

The ability of a tumour to spread is a fundamental property defining the malignant phenotype. NFκB regulates several genes associated with cellular invasion and metastasis, such as cell surface protease matrix metalloproteinases (*MMP-9*) (Bond *et al.*, 1998), urinary plasminogen activator (Novak *et al.*, 1991), and cell adhesion molecules including *ELAM-1, ICAM-1*and *VCAM-1* (Whelan *et al.*, 1991; Iademarco *et al.*, 1992; van de Stolpe *et al.*, 1994). Furthermore, it has been shown that inhibition of high constitutive NFκB activity using mutant IκBα decreased the frequency of metastases (Huang *et al.*, 2000; Fujioka *et al.*, 2003), indicating a potential role for aberrant NFκB signalling in tumour dissemination.

The role of NFκB in mediating programmed cell death is complex and may be pro- or anti-apoptotic depending on the environment and stimulus (Barkett and Gilmore, 1999). NFκB may contribute to growth of abnormal cells via up-regulation of anti-apoptotic genes such as cellular inhibitors of apoptosis (*cIAPs*) (You *et al.*, 1997; Stehlik *et al.*, 1998), members of Bcl2 family such as *Bcl-$_{XL}$* (Chen *et al.*, 2000) and down-regulation of pro-apoptotic genes including *Fas, caspase 11* and *TRAIL*. It is uncertain what determines whether NFκB activation is pro- or anti-apoptotic and it is the balance of signals, under a number of cellular and molecular scenarios, which determines the fate of any given cell.

It is clear that aberrations in NFκB signalling may confer a survival advantage on newly transformed cells destined to become malignant clones. However, there are conflicting data which indicate NFκB may also inhibit growth of squamous cell carcinoma in skin and that NFκB blockade triggers invasive human epidermal neoplasia (Shishodia and Aggarwal, 2004). A model reconciling these dichotomous NFκB roles in tumour promotion and suppression has been suggested. NFκB may act as a tumour suppressor in the early stages of tumorigenesis. Thereafter, accumulation of mutations in NFκB, NFκB-interacting genes or genes with κB promoter sequences, normally the target of NFκB regulation, results in loss of its tumour suppressor activity and the cancer promoting effects predominate (Perkins, 2004).

8.1.4 Evidence that aberrations of NFκB are involved in cancer

Evidence indicating oncogenic potential of NFκB originated from work on the avian retroviral protein v-Rel, a viral homolgue of c-Rel, which causes tumours in animal models (Gilmore, 1999), and c-Rel itself consistently transforms cells in culture (Gilmore *et al.*, 2004). Moreover, NFκB activation has been shown to result in cellular transformation and several oncogenes, such as *ras* and *c-myc*, mediate their effects through activation of NFκB (Aggarwal, 2004). Constitutive activation of NFκB in both haematological and solid malignancies may be promoting growth, since suppression of NFκB activity has been shown to inhibit cell proliferation, induce cell cycle arrest and apoptosis in tumours (Bharti and Aggarwal, 2002).

Rel A and *IkBa* genes are strong candidates since mutational activation or amplification of *Rel A* or upstream signals, or mutational inactivation of *IkBa* due to mutations in localisation or degradation sites may lead to aberrant NFκB signalling.

Possible explanations for high constitutive NFκB activity in tumours are firstly that IκBα is mutated and unable to mask the nuclear translocation signal in p65; secondly mutations in *Rel A* prevent IκBα binding or that the p65 upstream signal-transduction cascades are constitutively activated (Wang *et al.*, 1999b). Furthermore, functionally relevant mutations have been identified in constituents of the NFκB signalling pathway. *c-Rel, nfkb1, nfkb2* and *bcl-3* have been implicated in both haematological and solid tumour development through gene amplification and chromosomal rearrangement leading to overexpression (Rayet and Gelinas, 1999). Chromosomal rearrangements and amplifications of *Rel A* are less common in haematological malignancies (Trecca *et al.*, 1997). *Rel A* gene amplification has been observed in squamous head and neck, stomach and breast carcinoma and over-expression in thyroid cancer cell lines, but the role of *Rel A* in solid tumours is not fully defined (Rayet and Gelinas, 1999). The constitutive activation of NFκB in Hodgkin's lymphoma has been attributed to somatic mutations identified in the *IkBa* gene in Hodgkin's disease cell lines and patients (Wood *et al.*, 1998; Cabannes *et al.*, 1999; Krappmann *et al.*, 1999). Hence, there is substantial justification to study genetic alterations in *Rel A* and *IkBa* in colorectal cancer, given the key role of NFκB in aspirin-mediated anti-tumour effects presented in the preceding chapters.

8.1.5 Evidence for deranged NFκB signalling in colorectal cancer

Several lines of evidence indicate that NFκB signalling is abnormal in colorectal cancer. Altered regulation and increased expression of NFκB has been observed in the colorectal cancer cell line HT-29 and its metastatic counterpart HTM-29, specifically over-expressing p52 in the metastatic cells (Bours *et al.*, 1994). NFκB binding has been shown to be increased in colorectal cancers, compared with adjacent normal mucosa, and high constitutive NFκB activity has been observed in poorly differentiated and mucinous tumours (Lind *et al.*, 2001; Evertsson and Sun, 2002). Increased IKKα protein levels were observed, in association with increased NFκB expression, in tumour samples compared to matched normal colonic epithelium (Charalambous *et al.*, 2003). There is evidence that deranged NFκB signalling occurs early and is present in pre-malignant lesions, since increased NFκB expression has been observed in the stromal component of colorectal adenomas (Hardwick *et al.*, 2001). Furthermore, expression of p65 significantly increased in the transition from normal mucosa to adenoma and to adenocarcinoma (Charalambous *et al.*, 2003; Yu *et al.*, 2003). One of these studies also provides circumstantial evidence to suggest that increased expression of p65 is associated with a significant decrease in the apoptotic index (Yu *et al.*, 2003). Taken together, these studies strengthen the notion that increased NFκB expression and activity promotes tumourigenic behaviour in colorectal cancer.

NFκB may also determine the cellular response to chemotherapeutic agents (Ravi and Bedi, 2004). There was variability between rectal cancer patients in the magnitude of the

NFκB response to aspirin described in Chapter 7. This may be due to the baseline status of components of the NFκB pathway, which appear to be aberrantly active/expressed in some tumours and colorectal cancer cell lines. Interestingly, the patients with higher basal levels of IκB proteins were more responsive to NSAIDs in terms of IκB degradation and apoptosis.

Despite the substantial evidence discussed above suggesting that NFκB signalling is deranged in cancer, no mutations have been reported in key components of the pathway in colorectal cancer. The aim of this work was to determine *Rel A* and *IkBa* gene mutation prevalence in colorectal cancer using a panel of human colorectal cancers. Mutations in *Rel A* and *IkBa* in colorectal cancer are a plausible explanation for the variation in response to NSAIDs.

8.2 Overview of Methods

A 96-well plate was set up comprising DNA from patients with FAP, HNPCC, and sporadic colorectal cancers, including those from NSAID-treated patients in Chapter 7 and patient characteristics are described in Table 8.1. DNA from 5 of the colorectal cancer cell lines used in this study (SW480, HRT-18, HT-29, HCT-116 and LoVo), one positive control and 4 negative controls was also included using the methods described in Section 2.11 and 2.12.

Table 8.1 Patient characteristics of DNA for mutation analysis

Age	MSI-stable	MSI-low	MSI-high	FAP	NSAID group
10-20	3	-	1	-	-
20-30	4	2	4	1	-
30-40	4	4	4	1	-
40-50	4	-	4	-	3
50-60	5	2	3	-	2
60-70	4	-	4	-	6
70-80	5	2	3	-	4
>80	4	1	-	-	2
Total (n=86)	33	11	23	2	17

Mutation analysis comprised PCR for 10 exons in *Rel A* and 6 exons in *IkBa*, followed by automated sequencing on an ABI3700 as described in 2.12. Sequencher 3.0 software was used to analyse the chromatograms and sequence was compared to the published genomic sequence. Primers for both *Rel A* and *IkBa* genes were designed to include the intronic splice acceptor and splice donor sites to investigate any variants that may be modifying splicing. Hence, all variants were analysed using the following programs to check for generation of potential alternative splicing sites: ESE finder http://rulai.cshl.edu/tools/ESE/, BDGP Splice Site Prediction by Neural Network- http://www.fruitfly.org/seq_tools/splice.html and New GENSCAN Web Server at MIT - http://genes.mit.edu/GENSCAN.html.

8.3 Results

This work is currently ongoing and therefore the results are the most up to date at the time of writing this thesis. Nonetheless, the results presented here represent a sufficiently complete body of work to draw scientific conclusions. The conditions for the primers that were not working have been optimised and analysis of the remaining exons is underway.

8.3.1 Analysis of *Rel A* gene

Analysis of exons 3-7 and 10 of *Rel A* is shown in Table 8.2.

Table 8.2 Summary sequence analysis of *Rel A* gene

Rel A	PCR product size (bp)	Number sequenced	% Yield
Exon 1	231	--	--
Exon 2	343	34	37
Exon 3	254	36	39.1
Exon 4	218	80	87
Exon 5	259	81	88
Exon 6	203	82	89.1
Exon 7	330	86	93.5
Exon 8&9	330	--	--
Exon 10A	334	--	--
Exon 10B	291	63	68.5
Exon 10C	362	51	55.4

The variants identified to date in the *Rel A* gene are summarised below in Table 8.3.

Table 8.3 Variants identified in *Rel A* gene

Rel A	Number with variant	Number analysed	Change	Nucleotide	Codon	Predicted Changes
Intron 4	6	80	C→T	IVS4+27	-	unknown
Exon 7	1	86	del/ins	-	259	?frameshift
Exon 10B	1	63	G→C	1436	432	GGG-CGG Gly→Arg

The intronic C to T change at position IVS4 +27 was observed in 6 samples (7.5%)(Figure 8.3A). Analysis of matched normal DNA did not reveal the same change identified in tumour samples. This indicates the variant is a somatically acquired mutation with clonal amplification, suggesting it is functionally important. In exon 10, there was also a single nucleotide change (G→C, nucleotide 1436) at codon 432, which results in a change from a neutral non-polar to a basic polar amino acid (glycine to arginine) (Figure 8.3B). This may be important as it is within the second transactivation domain, which starts at codon 430. It is also near the phosphorylation site at T435 and close to the putative nuclear export sequence, which commences at codon 436 (Harhaj and Sun, 1999). A potential deletion/insertion was identified in exon 7 suggested by a double sequence extending 3' with normal sequence commencing at codon 259 (Figure 8.3C). At the time of writing, the potential deletion/insertion has yet to be identified and is currently undergoing further sequencing using forward and reverse primers to ensure the variant is not an artefact of sequencing compression. The wild-type and variant sequences were compared using the programs in section 8.2 to check if any of the variants create alternative splicing sites. There were no significant differences found suggesting that these changes do not disrupt splicing events.

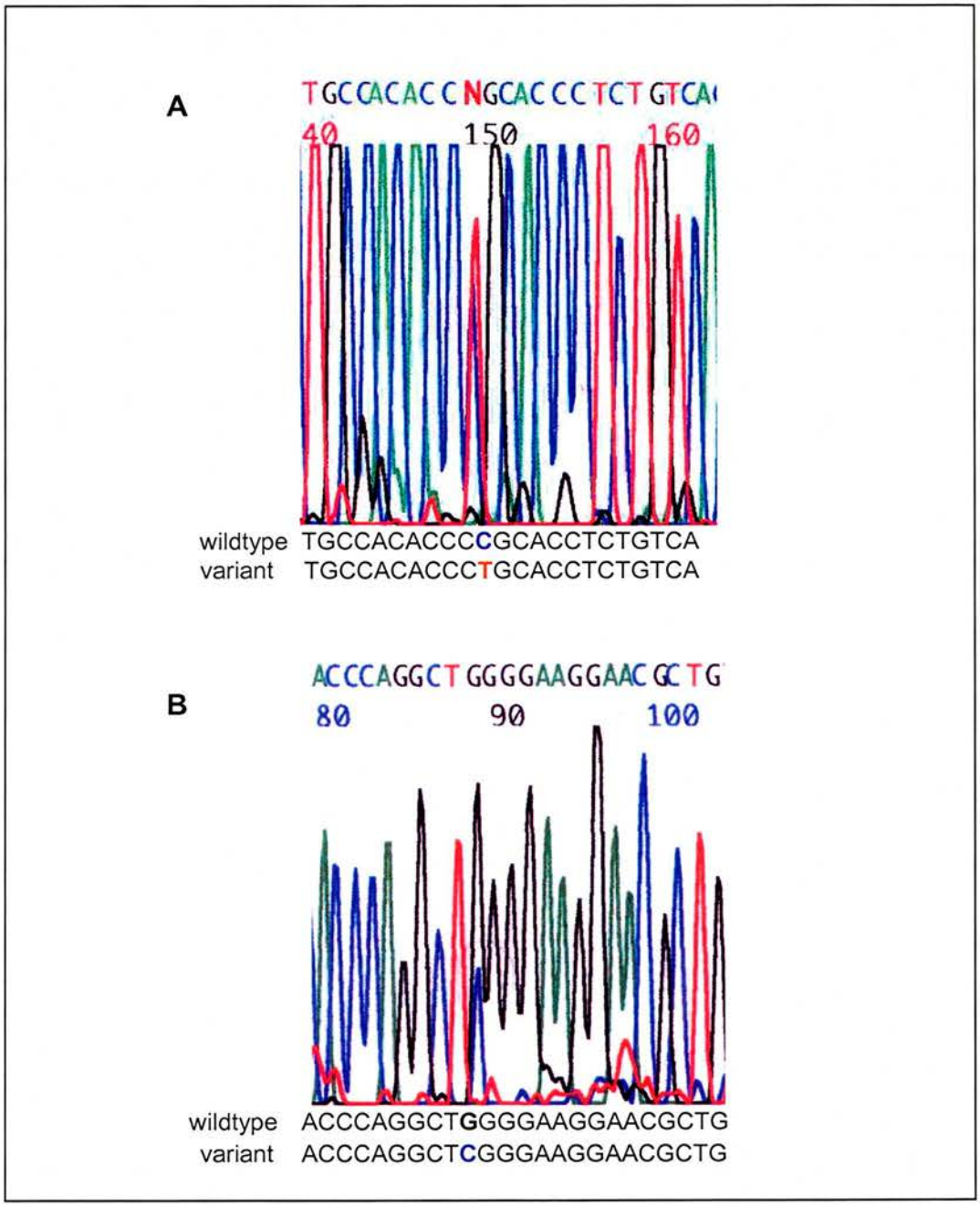

Figure 8.3 A & B *Rel A* variants
Intronic change (C to T) observed in 6 samples at IVS4+27(**A**).
Single nucleotide change (G→C) in exon 10B codon 432 (**B**).

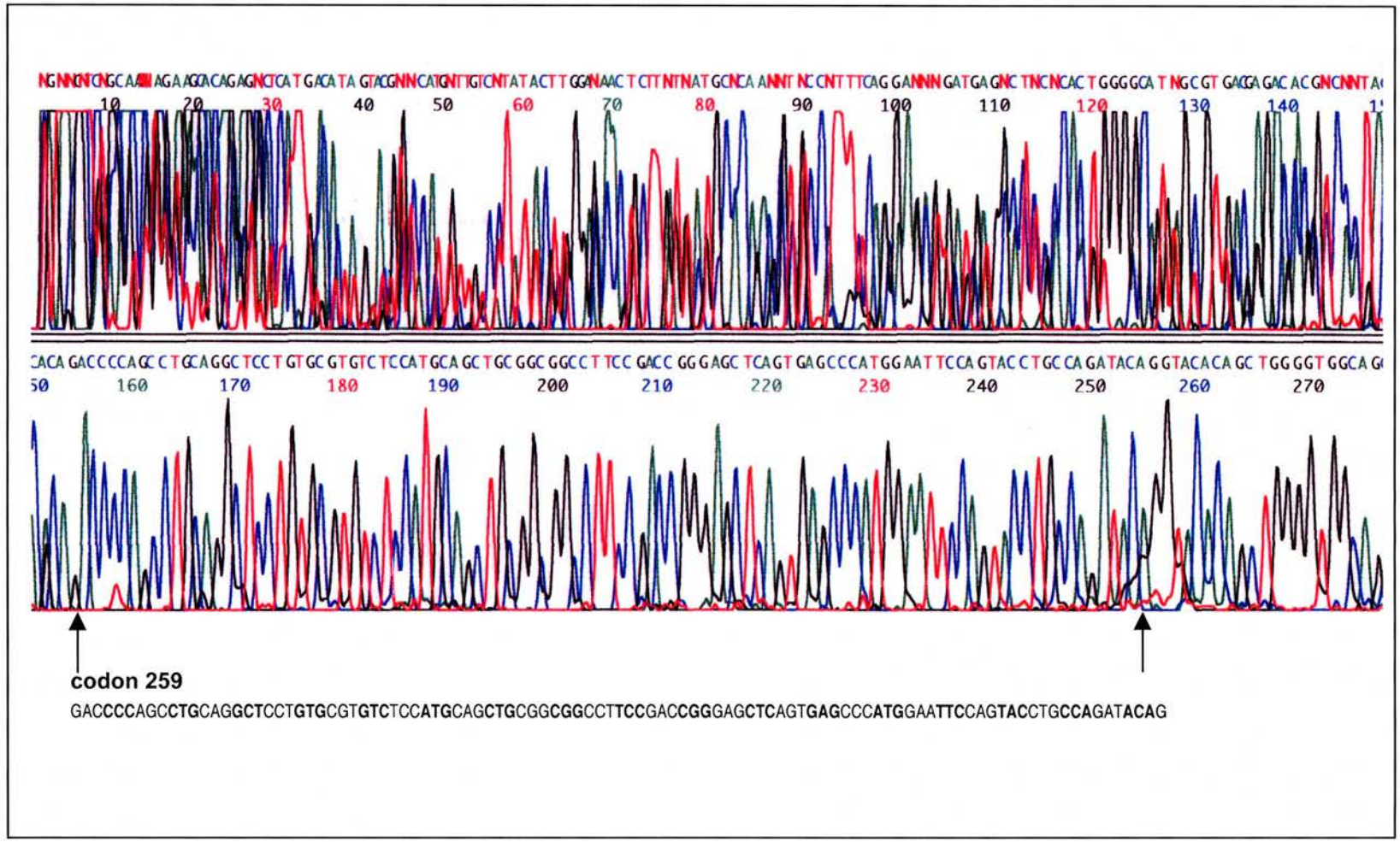

Figure 8.3 C *Rel A* variant exon 7
Possible deletion/insertion generating secondary sequence and then resuming normal sequence from codon 259.

8.3.2 Analysis of *IkBa* gene

Sequence analysis of exons 3-6 of the *IkBa* gene is summarised in Table 8.4 below.

Table 8.4 Summary of sequence analysis of *IkBa* gene

IkBa	PCR product size (bp)	Number sequenced	% Yield
Promoter	-	--	--
Exon 1A	346	--	--
Exon 1B	383	--	--
Exon 2	289	--	--
Exon 3	351	--	--
Exon 4	195	55	59.8
Exon 5	362	61	66.3
Exon 6	212	69	75

Two variants have been identified and are listed in Table 8.4.

Table 8.5 Variants identified in *IkBa* gene

IkBa	Number with variant	Number analysed	Change	Nucleotide position
Intron 4	36	55	C→T	IVS4 +17
3' UTR	46	69	T→C	3` UTR +2

There was a single nucleotide transition (C→T) at 17 nucleotides into intron 4 of *IkBa* observed in the 36 of 55 samples (65% frequency) (Figure 8.4A). This variant has been previously identified as a polymorphism in lymphoma patients (Jungnickel *et al.*, 2000), multiple myeloma patients (45% frequency) and in healthy individuals (46% frequency)

(Parker *et al.*, 2002; Rieder *et al.*, 2003). This appears to be a common single nucleotide polymorphism with heterozygous and homozygous genotypes identified (Figure8.4A).

There was also a nucleotide change (T to C) 2 nucleotides downstream from the stop codon in 46 samples (66% frequency)(Figure 8.4B). This variant has also been previously reported as a polymorphism since it was found not only in patients with lymphomas and multiple myelomas (frequency 83%) and but also in healthy individuals (frequency 33%) (Parker *et al.*, 2002; Rieder *et al.*, 2003).

It is recognised that alternative splicing of IκBβ may provide a means to selectively control the amount of IκBβ, bound to NFκB, to be released under NFκB-activating conditions (Hirano *et al.*, 1998). Hence, wild-type and variant *IkBa* sequences were compared using the programs in section 8.2 to determine whether any variants might influence splicing. No significant effects were predicted, suggesting that these changes do not result in splicing alterations. The prevalence might suggest these variants are simple polymorphisms.

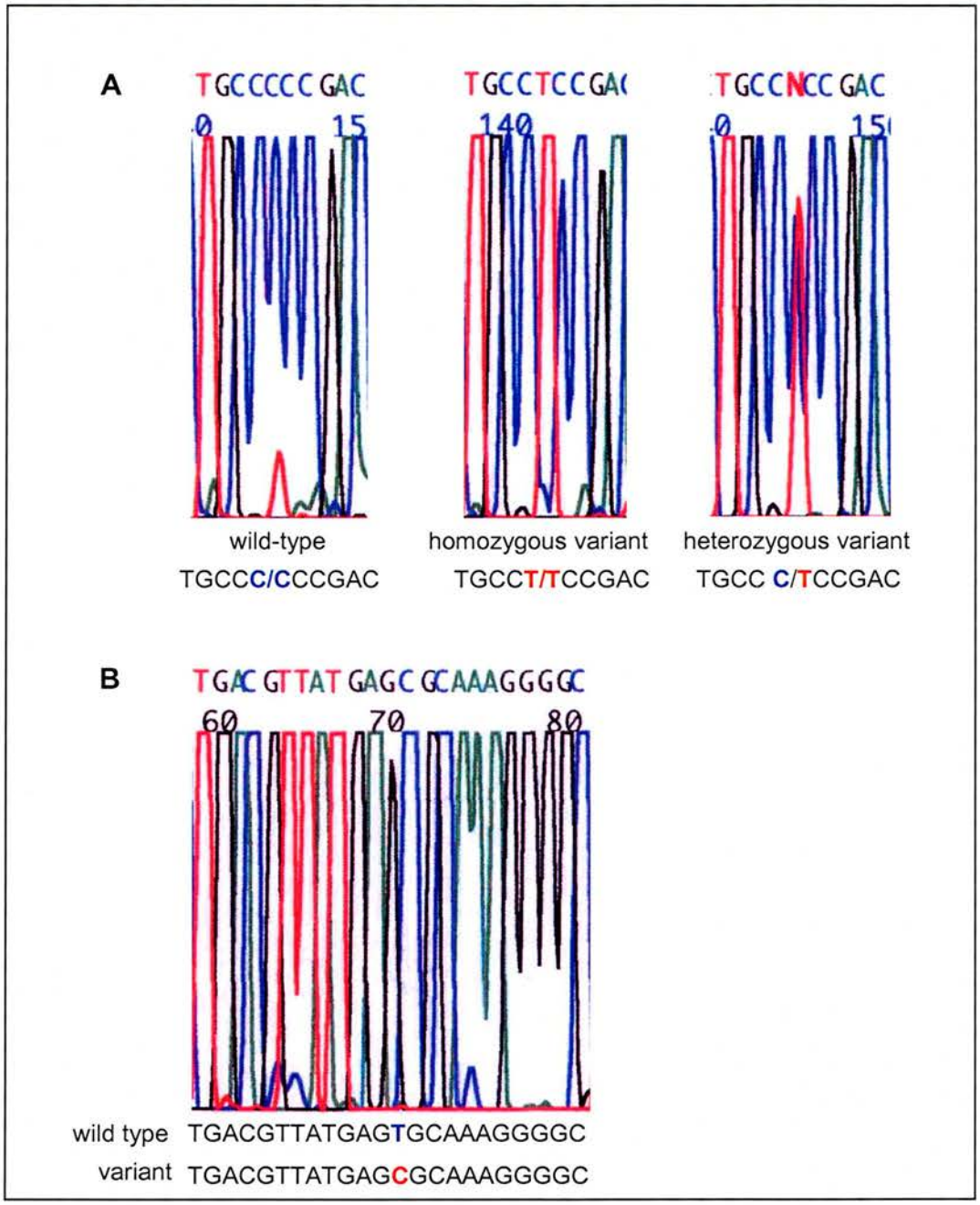

Figure 8. 4 *IkBa* variants
Single nucleotide transition (C to T) at IVS4+17 with heterozygous and homozygous genotypes (**A**). Single nucleotide change (T to C) at 3'UTR + 2 in 46 samples (**B**)

8.3.3 Polymorphisms and NSAID study patients

Polymorphisms are known to determine susceptibility to disease, but increasing evidence suggests that polymorphisms may determine response to prevention or intervention agents. There was no association between the *Rel A* and *IkBa* variants identified and response to NSAIDs, in terms of IκBα degradation (Table 8.6). However, the number of patients is not adequate to detect such any subtle effects of the variants.

Table 8.6 Variants and NSAID study patients

Patient ID	NSAID dose & type	*Rel A* IVS4+27	*IkBa* IVS4+17	*IkBa* 3' UTR+2	Response IκBα degradation
AB	300mg aspirin OD	WT	WT	**VAR**	Yes
SF		WT	WT	**VAR**	No
HG	600 mg aspirin OD	WT	WT	**VAR**	Yes
JS		WT	WT	**VAR**	No
MC		WT	WT	WT	Yes
DB	600 mg aspirin QDS	WT	WT	WT	Yes
JM		WT	WT	WT	Yes
JC		WT	**VAR**	WT	Yes
JL		WT	WT	**VAR**	No
ES		WT	**VAR**	-	Yes
DW	rofecoxib 25 mg OD	WT	**VAR**	-	Yes
AW		WT	WT	**VAR**	No
GW		WT	**VAR**	**VAR**	No
Frequency	-	0	31%	63.6%	-

WT- wild-type VAR-variant

-- sequencing failed

8.4 Discussion

The work presented in this chapter aimed to determine whether there are any DNA sequence variants of likely functional consequences in *Rel A* and *IkBa* genes in colorectal cancer. Increased expression and altered regulation of NFκB has been reported in colorectal cancer cell lines, adenomas and carcinomas (Bours *et al.*, 1994; Hardwick *et al.*, 2001; Lind *et al.*, 2001). High constitutive activity and aberrant NFκB signalling observed in colorectal cancer may be due to the presence of mutations in p65 and IκBα. The *Rel A* gene codes for p65 which is the transcriptionally active subunit of NFκB, and hence central for downstream regulation of NFκB-driven genes. Since mutations have been identified in IκBα, which through its inhibitory function plays a key role in regulating NFκB, it is essential to examine the *IkBa* gene for mutations.

8.4.1 *Rel A* and significance of variants

There is very little published data on mutation prevalence of *Rel A*. No mutations were found in *Rel A* both in HeLa cells and in HTM-29 cells, a metastatic cell line derived from colorectal cancer (Legrand-Poels *et al.*, 2000). Indeed, structural alterations of *Rel A* appear to be rare events in lymphoid neoplasia. In one case of multiple myeloma, a point mutation was identified resulting in an amino acid substitution at codon 494 (Glu to Asp) in the transactivation domain, which reduced its transactivation potential (Trecca *et al.*, 1997). Disruption of *Rel A* in mice results in massive liver cell apoptosis and embryonic lethality at 15 days of gestation (Beg *et al.*, 1995b). This suggests that defects in *Rel A* that disrupt growth and survival may be irreconcilable with cellular transformation, and hence constitutive activation of NFκB may select for tumour growth and survival (Rayet

and Gelinas, 1999). Indeed, this would support the notion that aspirin-mediated chemoprevention corrects disturbed mechanisms that permit escape from normal cellular turnover and apoptosis.

A number of variants of p65 that result in alternative splicing have been previously identified (Deloukas and van Loon, 1993). The first variant reported, p65Δ, is generated through an alternative splice acceptor sequence 30 nucleotides downstream of the native p65 splice acceptor site (Ruben *et al.*, 1992). The p65Δ protein lacks amino acids 222-231, necessary for p65/p50 interaction and for DNA binding. There is also a potential alternative acceptor splice site within intron 5, which may generate a longer variant of p65 due to the insertion of 19 amino acids between amino acids 187 and 188 (Deloukas and van Loon, 1993). Further p65 variants created by alternative splicing, p65Δ2 and p65Δ3 lack codons for amino acids 13-25 and 187- 293 respectively (Lyle *et al.*, 1994; Maxwell and Mukhopadhyay, 1995). Recognition of the alternative acceptor splice sites may be a regulated event and such splice variants of p65 may be responsible for selective transcriptional activity (Narayanan *et al.*, 1992).

The analysis of *Rel A* revealed a single nucleotide change (C to T) in 6 samples at 27 nucleotides into the fourth intron. This variant has previously been identified as having an allele frequency of 0.05 and an expected heterozygosity of 0.1 (Rieder *et al.*, 2003). Bioinformatics did not predict generation of alternative splicing sites due to this *Rel A* variant. It would have been useful to investigate transcripts of this variant from the respective tumours, but these were not available for analysis. However, this variant was only identified in 6 tumour samples and was not present in matched normal tissue, and

hence it is likely to be an acquired mutation with a prevalence of 7.5%. Further investigation of this variant is planned with fresh tumour samples, which would permit both RNA and protein extraction, to examine RNA transcripts and protein expression in order to determine functional significance.

Two exonic variants were identified in *Rel A*. There was a potential deletion/insertion in exon 7 which appeared to generate a secondary sequence and then resume normal sequence from codon 259 onwards. This variant may be important functionally due to its close proximity to the phosphorylation site at T254, which results in increased nuclear accumulation and stability of p65, and enhanced NFκB activity. The second variant identified was a single nucleotide change (G→C, nucleotide 1436) in exon 10 and results in a change from the amino acid glycine to arginine (codon 432). This variant is within the second transactivation domain of p65, which starts at codon 430. The variant may also affect phosphorylation at T435 by protein phosphatase 4 which results in inhibition of p65 activity (Yeh *et al.*, 2004). Lastly, the putative nuclear export sequence of p65 commences at codon 436 (Harhaj and Sun, 1999), and hence this change may affect p65 export resulting in increased nuclear accumulation and constitutive activation. Hence, the potential consequence of this variant may be increased NFκB transcriptional activity, either due to disruption of an inhibitory phosphorylation site or due to defective nuclear export.

8.4.2 *IkBa* and significance of variants

The role of IκBα in cellular transformation is not as well defined as that for NFκB. Anti-sense expression of *IkBa* transcripts have been shown to transform NIH3T3 cells suggesting that inactivation of IκB proteins promotes tumour development (Beauparlant *et al.*, 1994). Inactivation of the *IkBa* gene in mice results in neonatal fatality and hence precludes study of tumour development (Beg *et al.*, 1995a). Somatic mutations have been identified in *IkBa* in Hodgkin's disease, some of which result in loss of NFκB regulatory feedback, suggesting a tumour suppressor role (Krappmann *et al.*, 1999; Cabannes *et al.*, 1999; Jungnickel *et al.*, 2000). There is also some evidence that germline polymorphisms may be associated with a predisposition to multiple myeloma (Parker *et al.*, 2002).

In this chapter, *IkBa* mutation analysis has identified a number of variants in intron 4 and in the 3′ untranslated region. A single nucleotide transition (C to T) was identified at 17 nucleotides into intron 4, and this has been previously identified as a polymorphism (Jungnickel *et al.*, 2000). The estimated heterozygosity of this polymorphism is 0.21 (Rieder *et al.*, 2003). There was also a nucleotide change (T to C) present at 2 nucleotides after the stop codon in *IkBa*. This change has also been previously reported as a polymorphism and found in lymphomas, multiple myelomas and healthy controls (Jungnickel *et al.*, 2000; Parker *et al.*, 2002). The expected heterozygosity for this variant is 0.5 (Rieder *et al.*, 2003). This polymorphism is just beyond the stop codon and thus may be important for transcriptional regulation of *IkBa*. There is evidence that the 3'UTR of mRNA may modulate gene expression and disease susceptibility (Conne *et al.*, 2000). The size and prevalence of introns in human gene sequence (up to 99%), suggests introns could be important functional elements. For example, deletion of an intron in *hMLH1*

results in increased susceptibility to colorectal cancer (Farrington *et al.*, 1998). Hence, changes in both the intronic and untranslated regions of genes may have functional significance.

It is well established that polymorphisms may account for variation between individuals with respect to vulnerability to disease. An association between *COX-1* and *COX-2* polymorphisms and adenoma risk has been reported (Ulrich *et al.*, 2004; Ali *et al.*, 2005). The expanding field of pharmacogenomics is focussed on correlating therapeutic response to individual genetic profile. A study examining polymorphisms in cytokines and colorectal cancer risk in a Scottish population, found that carriers of the variant IL-10-592 (A) allele ingesting regular aspirin had a 50% reduced risk of colorectal cancer, compared to carriers of the A allele who did not use aspirin, or carriers with the CC genotype ingesting aspirin (Macarthur *et al.*, 2005). Polymorphisms in enzymes involved in aspirin metabolism, UGT1A6 and CYP2C9, have been shown to modify the effect of aspirin on colorectal adenoma development (Bigler *et al.*, 2001; Chan *et al.*, 2005). Although there was no apparent association between the *Rel A* and *IkBa* polymorphisms and response in the NSAID study, the numbers required to detect variations would be substantial. It is likely that subsets of the population defined by genotype have a differential response to chemopreventive agents.

Mutation analysis has identified a number of potential functionally important variants. The variant in *Rel A* at codon 432 may be functionally relevant as it is in the transactivation domain of the protein. The *Rel A* intronic variant was only identified in tumour tissue suggesting it is an acquired mutation. The polymorphisms in *IkBa* have

been previously identified but may play a role in determining disease susceptibility and response to agents. Immunohistochemistry will be carried out to investigate whether protein expression is altered. In addition, RT-PCR analysis would be performed to determine the effects of variants on RNA transcripts. This work will continue with sequence analysis of the remainder of the *Rel A* and *IkBa* genes. This research will inform whether there is a genetic component contributing to deregulated NFκB signalling in colorectal cancer.

Chapter 9

Summary Discussion

Worldwide, there are 500,000 new cases of colorectal cancer per annum. There has been some recent improvement in mortality but overall 5-year survival is 51%. Research endeavour has begun to focus on early detection and prevention using drugs that intervene in tumour development. The ideal chemopreventive agent should inhibit carcinogenesis and influence tumour biology, have an acceptable toxicity profile, and should possess differential activity between normal and abnormal epithelium (Thiery-Vuillemin *et al.*, 2005). With respect to these criteria, NSAIDs fulfil the requirement as potential chemopreventive agents in colorectal cancer. Despite the 40-50% risk reduction in colorectal cancer, the safety profile of NSAIDs is a relative barrier to widespread use as primary chemopreventive agents. Hence, there is compelling rationale to define the molecular basis of the protective effect of NSAIDs in colorectal cancer, in order to target the mechanism of action precisely. The work presented in this thesis contributes to the understanding of the mechanism of NSAID-mediated anti-tumour activity via modulation of NFκB signalling in colorectal cancer.

The NFκB signalling pathway is critical in several cellular processes such as proliferation, invasion and death, which may influence tumour development and progression. Genes regulated by NFκB, including *COX-2* and *p53*, and NFκB signalling

itself are deregulated in colorectal cancer. Indeed there appears to be progressively aberrant expression of NFκB from adenomas to carcinomas, indicating that NFκB plays a key role in colorectal carcinogenesis (Yu *et al.*, 2003). The initial findings of Stark and Dunlop in the host laboratory identified a novel mechanism of action underlying aspirin-induced apoptosis in colorectal cancer cells (Chapter 3). This work showed that aspirin induces apoptosis in colorectal cancer cells that is paralleled by IκBα degradation and NFκB nuclear translocation in a dose-dependent manner. The effects of aspirin on NFκB and apoptosis were totally abrogated in colorectal cancer cells engineered to express mutant IκBα that is resistant to degradation. Previous work had indicated that aspirin inhibited NFκB by decreasing IKKβ activity, following TNFα stimulation of the NFκB signalling pathway (Yin *et al.*, 1998; Yamamoto *et al.*, 1999b). Chapter 3 describes the effects of aspirin on NFκB signalling and apoptosis in the *absence* of stimulating agents such as TNFα, which more accurately reproduces colorectal epithelial exposure to NSAIDs. The effects on NFκB are evident at low doses of aspirin that are realistic in humans, and hence relevant to chemoprevention.

The importance of NFκB as a central target for aspirin-mediated apoptosis, relevant particularly to colorectal cancer, is further highlighted by the work presented in Chapter 4. This demonstrated a striking difference in the response to aspirin between colorectal cancer cell lines and lines derived from breast, ovarian and endometrial cancers, with respect to both cell viability and NFκB signalling. Aspirin-induced apoptosis, associated with IκBα degradation and NFκB nuclear translocation, was restricted to colorectal cancer cell lines. The relationship between aspirin-induced apoptosis and the effects on NFκB signalling, suggests a molecular rationale for the greater sensitivity of colorectal

cancer to NSAIDs compared to other cancers. These results provide mechanistic support for the epidemiological observations that NSAIDs provide greater protection from colorectal cancer compared to other cancers.

In addition to demonstrating that the effect of aspirin on NFκB signalling and apoptosis is relatively specific for colorectal cancer, the data in Chapter 4 show that this effect occurs in a wide range of colorectal cancer cell lines. These colorectal cancer cell lines have varying genetic backgrounds emphasising the *generality* of the aspirin-induced NFκB apoptotic response in colorectal cancer. The data strongly suggest that the aspirin-induced NFκB apoptotic effect is likely to confer broad preventive activity against colorectal cancer. Nonetheless, it would be important to identify whether there are subsets of colorectal cancers that are more or less susceptible to NSAID-mediated chemoprevention. Indeed, identification of molecular markers that predict response with respect to NSAID-mediated chemoprevention and adjuvant therapy would be informative.

In the literature, the COX-2 enzyme has been implicated as both a molecular target of NSAIDs and a potential marker of response. However, the results presented in Chapter 5 demonstrate that COX-2 expression levels are unrelated to susceptibility to aspirin-induced apoptosis via NFκB modulation. This is consistent with substantial evidence suggesting that COX-2 is not the predominant mechanism for the anti-tumour effects of NSAIDs. By using colorectal cancer cell lines that are similar other than a specific genetic change under investigation, the results presented in Chapter 6 show that colorectal cancer cells undergo apoptosis, following IκBα degradation and NFκB nuclear

translocation, in a manner that is independent of p53 and MMR mutation status. The results from Chapters 4 and 5 show colorectal cancer cells are sensitive to aspirin-mediated apoptosis independent of *APC* mutation status and β-catenin expression. This is particularly relevant since aberrant *Wnt* signalling is the earliest molecular derangement in colorectal tumorigenesis. Hence, it is evident that the aspirin-induced NFκB apoptotic response occurs irrespective of early (*APC*, β-catenin, COX-2), intermediate (MMR) and late (p53) mutational events in colorectal cancer development. These results emphasise that aspirin has potential to prevent all subtypes examined irrespective of such molecular events, and imply that aspirin could target all colorectal neoplastic cells as they arise. This is important when considering translating these findings to clinical studies aimed at defining the NFκB response to aspirin in human colonic epithelium and tumours.

It was essential to ensure that the aspirin-induced NFκB apoptotic response predicts that identifiable groups at high risk of colorectal cancer, with currently definable genetic defects, could potentially benefit from chemoprevention. Indeed, the risks associated with chemoprevention would be balanced against the higher risk of developing colorectal cancer in genetically predisposed groups such as HNPCC and FAP. The results from Chapter 4 and 6 show that colorectal cancer cell lines with mutations in DNA MMR genes are all susceptible to aspirin-induced apoptosis. This data is relevant not only to prevention but also to potential therapeutic use since MMR status has been shown to confer resistance to certain chemotherapy. The finding that the aspirin-induced NFκB response is not influenced by *APC* mutation status and aberrant β-catenin expression is relevant to patients with FAP. Furthermore, the clinical studies presented in Chapter 7 suggest that there may be evidence of modulation of NFκB in both HNPCC and FAP

patients. From a prevention standpoint in genetically predisposed groups, the studies of HNPCC and FAP could be instructive as to whether modulation of NFκB signalling in normal mucosa of such patients can be demonstrated. However, it is unclear whether any patients responded to NSAIDs, manifest as changes in IκBα levels, because of the variability in IκBα levels. Possible explanations include variations in therapeutic levels, pharmacokinetic, and pharmacodynamic differences and also the possibility that specific somatic genetic events other than the causative mutation may impact on NSAID response. This must remain speculative as elucidating the underlying reason for differential response in high-risk groups and in average risk groups lies outwith the remit of this thesis.

Having identified a novel molecular mechanism of action *in vitro*, it was key to investigate whether this translated into an effect in patients at moderate risk of colorectal cancer. The fraction of basic scientific research making a successful transition to clinical use or benefit is limited. For example, the impact of the EGFR inhibitor gefitinib on overall lung cancer survival in clinical trials was disappointing compared to that predicted from the encouraging molecular work (Comis, 2005). The research presented in Chapter 7 is the first to examine the effects of NSAIDs on NFκB signalling and apoptosis in patients with established rectal cancer. The results show that changes in IκBα expression and apoptosis are detectable rectal cancer patients but there is high level of variability and further experiments are required to define the intra-patient variability of the protein and the effects in untreated patients. Subsequent experiments, in addition to the work that constitutes this thesis, have shown that aspirin does indeed induce p65 nuclear translocation in these rectal cancer patients (Din *et al.*, 2005). The effect of NSAIDs on

NFκB signalling *in vivo* is supported by research in two mouse models which shows that aspirin induces cytoplasmic IκBα degradation, p65 nuclear translocation and cleavage of caspase-3 in xenografted HT-29 tumours and in adenomas from $Apc^{Min+/-}$ mice (Stark *et al.*, 2006).

Activation of the NFκB pathway normally results in increased NFκB transcriptional activity. However, aspirin-induced nuclear translocation of NFκB has been shown to *repress* NFκB transcriptional activity in colorectal cancer cell lines (Stark *et al.*, 2000). This work showed that aspirin induces NFκB nuclear translocation and repression of NFκB-driven transcription, which is not a general inhibitory effect on signalling pathways or transcriptional machinery. Studies into the kinetics of this response demonstrated that IκBα degradation and NFκB nuclear translocation precede repression of NFκB-driven transcription, commensurate with a causal relationship. Furthermore, inhibition of NFκB nuclear translocation by super-repressor IκBα inhibited aspirin-mediated repression of NFκB transcriptional activity. The data from Chapter 6 show that p53 and MMR mutation status do not affect the property of aspirin to induce NFκB transcriptional repression in colorectal cancer cells. Indeed, it has been shown that NFκB induced by some cytotoxic stimuli acts as a *repressor* of anti-apoptotic gene expression (Campbell *et al.*, 2004). There is increasing evidence that NFκB can act as an activator or repressor of transcription, dependent on the nature of stimulus and cell environment. Recently, the host laboratory further elucidated the mechanism underlying repression occurring as a consequence of p65 nuclear translocation by demonstrating that aspirin induces nucleolar sequestration of p65, the transcriptionally active subunit of NFκB (Stark and Dunlop, 2005). Furthermore, identification of a nucleolar localisation signal

(NoLS) at the N-terminus of p65 and use of a dominant negative mutant of p65 shows that aspirin-induced nucleolar sequestration of p65 is responsible for decreased basal NFκB transcriptional activity and apoptosis. This suggests that aspirin might cause repression of NFκB-driven anti-apoptotic genes and such studies are currently underway.

It is vital to investigate the effects of aspirin on NFκB activity and effects on relevant downstream genes. In this group of rectal cancer patients, aspirin has been shown to decrease expression of the NFκB-driven anti-apoptotic gene BCL-$_{XL}$ and increase expression of the pro-apoptotic BAX gene in rectal tumours (Macdonald ADH *et al.*, 2004; Macdonald ADH *et al.*, 2005b). Furthermore, in these aspirin treated rectal cancer patients, there is decreased expression of the cell adhesion molecule ICAM-1, which is known to be deranged in colorectal cancer and be involved in metastasis (Macdonald ADH *et al.*, 2005a). These results suggest that NSAIDs are affecting expression of NFκB-driven genes *in vivo*. The next phase will involve determining which downstream genes are relevant to the apoptotic response, and defining the balance between pro- and anti-apoptotic genes.

Constitutive NFκB activation is known to permit escape from apoptosis and to be associated with resistance to chemotherapeutics. Such activation may be due to alterations in the NFκB pathway genes, uncontrolled IKK stimulation or constitutive activation of other upstream kinases. Mutations and deletions in *IkBa* have previously been observed in lymphomas, which also show constitutive activation of NFκB. The work presented in Chapter 8 screened a panel of colorectal cancers for mutations and polymorphic variants

in *Rel A* and *IkBa* genes. This analysis has identified somatic mutations and other variants that merit further study. Regions of both the *Rel A* and *IkBa* genes, highly relevant to function of the respective proteins, have not been fully sequenced yet but completion of analysis of both genes is ongoing. The variant in *Rel A* at codon 432 is in the transactivation domain and may be functionally relevant. The *Rel A* intronic variant is highly likely to be important, as it is an acquired mutation only identified in tumour tissue. It will also be important to determine whether the *IkBa* polymorphisms play a functional role in determining susceptibility to colorectal cancer or response to NSAIDs. This work will inform whether there is a genetic component contributing to deregulated NFκB signalling in colorectal cancer.

There are other potential NFκB pathway candidate genes such as upstream kinases that may be responsible for continual activation of the NFκB pathway. A recent analysis of human colorectal cancers for genetic mutations in 340 serine/threonine kinases revealed mutations in eight genes, including three constituents of the phosphatidylinositol-3-OH kinase (PI3K) pathway (Parsons *et al.*, 2005). Overall 40% of colorectal cancers had a mutation in at least one or more of the eight PI3K genes. The PI3K pathway is involved in cell survival and is negatively regulated by the tumour suppressor gene PTEN (phosphatase and tensin homologue) (Di Cristofano and Pandolfi, 2000). This may be important since NFκB has been shown to downregulate expression of PTEN and prevent apoptosis (Vasudevan *et al.*, 2004). It has been reported that NSAIDs activate PTEN in colorectal cancer cell lines (Chu *et al.*, 2004). Hence, it is plausible that NSAID-mediated repression of NFκB results in increased expression of PTEN and apoptosis.

Research in prevention and treatment of cancer is advancing and molecular targeted therapies are rapidly evolving into clinical practice, including Her-2 in breast cancer, BCR-ABL in chronic myeloid leukaemia, C-KIT in gastrointestinal stromal tumours, and EGFR in lung cancer (Thiery-Vuillemin *et al.*, 2005). Such molecular targeted treatment confers a greater degree of specificity than standard empirical therapy. NSAIDs are emerging as important chemopreventive agents and also possibly as therapeutic agents against colorectal cancer. It is clear that NSAIDs have a number of different cell and molecular effects, and the observed relative cell-type specificity suggests that distinct pathways might be targeted by aspirin in colorectal cancer cells. The research in this thesis demonstrates that aspirin induces apoptosis following modulation of the NFκB pathway, providing evidence of a molecular rationale for the greater specificity of NSAID-mediated protection observed in colorectal cancer compared to other cancers. The lack of dependency on COX-2 and β-catenin expression, and independence from MMR and p53 mutation status emphasises the generality of the aspirin-induced NFκB apoptotic response in colorectal cancer. Establishing the molecular basis of patient variability in response to aspirin would be a major advance, since currently available agents could be targeted to people who are at risk of colorectal neoplasia but also based on whose response could be predicted from molecular markers. Indeed, the validity of investigating chemopreventive mechanisms of action in cancer tissue may be called into question. However, it is not feasible to study molecular mechanisms of prevention in normal colonic tissue given the time-frame involved in development to cancer. The advantage of studying cancer cells is that it informs which molecular pathways NSAIDs may be affecting. This permits study of individual deranged pathways in colorectal cancer including early aberrations in *Wnt* signalling and late events such as p53. Indeed, it is the investigation of NSAIDs as chemopreventive agents in cancer cells that has brought to

light their therapeutic potential. With respect to prevention, the host laboratory has shown that aspirin suppress tumorigenesis in $Apc^{Min/+}$ and $Apc^{Min/+}$, $Msh2^{-/-}$ mice (Sansom *et al.*, 2001). Indeed, the effect was only seen when animals were exposed to aspirin *in utero*, suggesting aspirin affects neoplastic transformation at the very earliest stages of development. Unfortunately, p65 null animals are non-viable, but it would be interesting to investigate the specific role of nucleolar targeting of NFκB using *in vivo* models. Thus, the effects of aspirin on xenografted tumour cells stably expressing p65 lacking the NoLS (Stark and Dunlop, 2005) could be compared to cells expressing wild-type p65. Another approach would be to generate a genetically modified animal in which wild-type p65 has been replaced by an exogenous p65 that lacks the NoLS, through germline targeting. The phenotype of animals expressing the p65-NoLS$^{-/-}$ would be of considerable interest, and in particular when crossed with Apc^{Min} animals and treated with aspirin. This might require generating a conditional knockout using cre-recombinase technology given the embryonic lethality of p65 null animals.

The central role of NFκB signalling as a key target in NSAID-mediated apoptosis is supported and substantially strengthened by the cell line data presented in this thesis. The concept of chemoprevention is based upon intervening in the tumourigenesis process by which a normal cell acquires defects, which permit clonal expansion of abnormal cell populations. Hence, it may be that NSAIDs prevent tumour cell expansion by shifting the balance towards apoptosis via NFκB modulation of apoptosis-related genes. It may also be that other NFκB related effects such as inhibition of angiogenesis contribute to the anti-tumour activity. The NFκB pathway is complex and modulation of signalling activates numerous pathways that positively and negatively regulate survival. The

challenge lies in identifying which specific components may be targeted to shift the balance towards eliminating tumour cells. The research presented in this thesis contributes to understanding the molecular mechanism underlying NSAID-mediated anti-tumour activity in colorectal cancer and hopefully will inform future work towards novel drug design.

Chapter 10

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Appendix I: Presentations

1. University of Edinburgh School of Surgery Day, November 1999, Edinburgh

Molecular Basis of the Antineoplastic Activity of Aspirin in Colorectal Cancer

FVN Din, LA Stark and MG Dunlop

2. Genes and Cancer Meeting, December 1999, Warwick

Aspirin-mediated IκB Degradation and Apoptosis in Colorectal Cancer (poster)

FVN Din, LA Stark and MG Dunlop

3. Association of Coloproctology: Scottish Chapter Meeting, March 2000, Stirling

Molecular Basis of the Anti-tumour Activity of Aspirin in Colorectal Cancer

FVN Din, LA Stark and MG Dunlop

4. American Association of Cancer Research, April 2000, San Francisco

Aspirin-induced IκBα degradation & apoptosis in colorectal cancer cell lines (poster)

FVN Din, MG Dunlop and LA Stark

5. Surgical Research Society, October 2000, Cork

Molecular Basis of Aspirin-induced Apoptosis in Colorectal Cancer

FVN Din, LA Stark and MG Dunlop

6. Scottish Society for Experimental Medicine, October 2000, Edinburgh

Aspirin and Colorectal Cancer Chemoprevention

FVN Din, LA Stark and MG Dunlop

7. 4th Nottingham International Colorectal Cancer Symposium, October 2001

Aspirin Effects on NFκB & Apoptosis: Evidence for Colorectal Cancer Specificity

FVN Din, LA Stark and MG Dunlop

8. Association of Coloproctology, Manchester, July 2002

Aspirin Effects on NFκB & Apoptosis: Independence from MMR & p53 Status

FVN Din, LA Stark and MG Dunlop

9. Association of Coloproctology, Edinburgh, July 2003

Aspirin Effects on NFκB & Apoptosis:Evidence for Colorectal Cancer Cell Specificity

FVN Din, LA Stark and MG Dunlop

10. Clinical & Scientific Meeting Royal College of Surgeons Nov 2003 Edinburgh

Surgeon-in-Training Medal Session: Aspirin-Induced Apoptosis Following NFκB Nuclear Translocation is not influenced by Mismatch Repair and p53 Status

FVN Din, LA Stark and MG Dunlop

11. Society of Academic and Research Surgery, Belfast, January 2004

Aspirin Induced Apoptosis And Effects On NFκB Responsive Genes, Bax and Bcl-X_L

A Macdonald, F Din, L Stark and M Dunlop

12. Keystone Symposia: NFκB, Utah, January 2004

Aspirin Induced Apoptosis & Effects On NFκB Responsive Genes, Bax and Bcl-X_L

A Macdonald, F Din, L Stark and M Dunlop (poster)

13. Cancer Research Centre Symposium, Edinburgh, February 2004

Aspirin Effects on NFκB & Apoptosis: Independence from DNA MMR & p53 Status

FVN Din, LA Stark and MG Dunlop

14. Int. Society for Hereditary Gastrointestinal Tumours, Newcastle June 2005

Aspirin-induced nuclear translocation of NFκB & apoptosis in colon cancer is independent of p53 mutation status & MMR proficiency

FVN Din, LA Stark & MG Dunlop

15. Royal College of Surgeons' Quincentenary Meeting Edinburgh, July 2005

NSAIDs Induce Activation of NFκB & Apoptosis in Rectal Cancer Patients

FVN Din, LA Stark AND MG Dunlop

16. Royal College of Surgeons' Quincentenary Meeting, Edinburgh, July 2005

Aspirin Represses NFκB-driven Transcription of Bcl-X_L Gene in Colorectal Cancer

A Macdonald, F Din, L Stark and M Dunlop

17. Association of Coloproctology Tripartite Meeting Dublin, July 2005

Aspirin Represses NFκB-driven Transcription of ICAM-1 Gene in Colorectal Cancer

A Macdonald, F Din, L Stark and M Dunlop (poster)

18. Association of Coloproctology Tripartite Meeting Dublin, July 2005

NSAIDs Induce Apoptosis via NFκB Modulation in Colorectal Cancer Patients

FVN Din, LA Stark AND MG Dunlop

19.Wilfrid Card Lecture, Edinburgh, November 2005

Colorectal Cancer Chemoprevention: 'Aspirin'g towards Apoptosis via NFκB signalling

FVN Din

Appendix II: Publications

Original articles

1. Aspirin-induced activation of the NF-κB signalling pathway: a novel mechanism for aspirin-mediated apoptosis in colon cancer cells.

FASEB J. 2001; 15(7):1273-5.

LA Stark, FVN Din, RM Zwacka and MG Dunlop

2. Evidence for colorectal cancer cell specificity of aspirin effects on NF kappa B signalling and apoptosis

British Journal of Cancer. 2004 Jul 19; 91(2): 381-8

FVN Din, MG Dunlop and LA Stark

3. Aspirin-induced apoptosis following NFκB nuclear translocation is not influenced by mismatch repair and p53 Status.

British Journal of Cancer 2005 92:1137-43

FVN Din, MG Dunlop & LA Stark.

4. Aspirin activates the NFκB signalling pathway and induces apoptosis *in vivo*, in two animal models of colorectal cancer

Accepted Carcinogenesis

LA Stark, K Reid, O Sansom, FV Din, S Guichard, DI Jodrell, A Clarke & MG Dunlop

Published Peer Reviewed Abstracts

1. Aspirin-induced IκBα degradation and apoptosis in colorectal cancer cell lines.

Proceedings of the American Association of Cancer Research 2000 Volume 41,495

FVN Din, MG Dunlop and LA Stark

2. Aspirin induced apoptosis of colorectal cancer cells is caused by nuclear translocation of repressive NFκB complexes

Proceedings of the American Association of Cancer Research 2000 Volume 41, 495

LA Stark, FVN Din and MG Dunlop

3. Molecular basis of aspirin-induced apoptosis in colorectal cancer.

British Journal of Surgery 2001, Volume 88, (5): 755-756

FVN Din, LA Stark and MG Dunlop

4. Aspirin-Induced Apoptosis Following NFκB Nuclear Translocation Is Not Influenced by Mismatch Repair and p53 Status

Colorectal Disease Supp. 2002 4 1:11

FVN Din, MG Dunlop & LA Stark

5. Aspirin Effects on NFκB Signalling & Apoptosis: Evidence for Colorectal Cancer Cell Specificity

Colorectal Disease Supp. 2003 5 1:22-23

FVN Din, LA Stark and MG Dunlop

6. Aspirin Induced Apoptosis And Effects on NFκB Responsive Genes, Bax & Bcl-$_{XL}$

SARS Yearbook 2004 (http://www.surgicalresearch.org.uk/pdf%27s/FINALSARS.pdfAbstract 19, pg 44,

A Macdonald, F Din, L Stark and M Dunlop

7. Aspirin-Induced Nuclear Translocation of NFκB and Apoptosis in Colorectal Cancer is Independent of p53 Status and Mismatch Repair Proficiency

Familial Cancer Supp 2005 4 (Supp 1): 57

FVN Din, LA Stark and MG Dunlop

8. Aspirin Represses NFκB driven Transcription of Bcl-$_{XL}$ gene in colorectal cancer

Journal of the Royal Colleges of Surgeons Supp 2005 3 3:S19

A Macdonald, F Din, L Stark and MG Dunlop.

9. NSAIDs Induce Activation of NFκB & apoptosis in Rectal Cancer Patients

Journal of the Royal Colleges of Surgeons Supp 2005 3 3:S6

FVN Din, LA Stark and MG Dunlop

10. Aspirin represses NFκB driven transcription of ICAM-1 in colorectal cancer

Colorectal Disease Supp. 2005 7 1:57

A Macdonald, F Din, L Stark and MG Dunlop.

11. NSAIDs Induce Apoptosis via NFκB Modulation in Colorectal Cancer Patients

Colorectal Disease Supp. 2005 7 1:5

FVN Din, LA Stark and MG Dunlop

Appendix III: Prizes

Chiene Medal in Surgery

School of Surgery Day

Molecular basis of the antineoplastic activity of aspirin in colorectal cancer

November 1999

Sir James Black Prize -1st prize

Scottish Society for Experimental Medicine

Aspirin and colorectal cancer chemoprevention

October 2000

Colon and Rectal Disease Research Foundation Prize

Association of Coloproctology of Great Britain and Ireland

Aspirin Effects on NFκB Signalling & Apoptosis: Independence from DNA MMR & p53

July 2002

Wilfred Card Lectureship

University of Edinburgh

Colorectal Cancer Chemoprevention: 'Aspirin'g towards Apoptosis via NFκB signalling

November 2005

Appendix IV: Papers

The FASEB Journal express article 10.1096/fj.00-0529fje. Published online March 20, 2001.

Aspirin-induced activation of the NF-κB signaling pathway: a novel mechanism for aspirin-mediated apoptosis in colon cancer cells

L.A. Stark, F.V.N. Din, R.M. Zwacka, and M.G. Dunlop

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ABSTRACT

The use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a lower risk of colorectal cancer. However, the underlying mechanism is complex and remains to be fully elucidated. NSAIDs have modulated NF-κB in response to stimulating cytokines, but the link between NF-κB and aspirin-induced growth inhibition of colorectal cancer cells has yet to be thoroughly investigated. In the present study, we examined the effects of aspirin on the NF-κB pathway and the association between these effects and apoptotic tumor cell death. We found that aspirin induced a concentration and timedependent reduction in cytoplasmic IκBα in colorectal cancer cells that was due to phosphorylation-dependent, proteosome-mediated degradation of the protein. With aspirin-induced IκBα degradation, we observed nuclear translocation of NF-κB, as determined by immunocytochemistry and electrophoretic mobility shift assays (EMSAs). The NF-κB response to aspirin preceded cell death and was, therefore, more likely to be a cause than a consequence. To further investigate this relationship, we generated HRT18 and CT26 colon cancer cells that constitutively expressed a super-repressor IκBα. We found that inhibition of NF-κB nuclear translocation in these cells abrogated aspirin-induced apoptosis. When we investigated the cell specificity of the aspirin-induced response, we observed both IκBα degradation and growth inhibition in a panel of colorectal cancer cells, but there was no effect in cells of noncolonic origin. Thus we conclude that aspirin induces activation of NF-κB, which is required for its anti-tumor activity and may contribute to the protective effect of aspirin that has been observed in clinical trials.

Key words: NF-kappaB • colorectal cancer • NSAIDs • IκBα • chemoprevention

An estimated 250,000 people are diagnosed with colorectal cancer annually in the United States and Europe (http://www.aacr.org/), and 500,000 people are diagnosed worldwide. Half of these patients have metastatic disease at initial presentation, and current conventional chemotherapies impart only marginal survival benefit (1). Hence, much effort is focused on developing strategies for early detection and prevention. One class of compounds that

have shown promise in the prevention of colon cancer are aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs) (2–6). . NSAIDs have inhibited the growth of colorectal cancer cells *in vitro* (7–11) , inhibited tumor formation in the colons of carcinogen treated rodents (12–14), and reduced adenoma counts in humans with familial adenomatous polyposis (15–18). The most compelling evidence that NSAIDs prevent colon cancer comes from epidemiological data indicating that people who regularly take NSAIDs have a 40–50% reduced risk of dying from this disease compared with matched controls (2, 17, 19). However, the detrimental side effects of NSAIDs limit their potential as chemopreventative agents (20). Therefore, to allow development of safer alternatives, we need to understand the mechanisms by which NSAIDs exert their chemopreventative effects. These mechanisms appear to be complex and have yet to be fully elucidated.

There is a body of literature that suggests that the antineoplastic activity of NSAIDs is predominantly mediated by inhibition of the cyclooxygenase (COX) enzymes (4, 5, 21). Consistent with this, overexpression of COX-2 is associated with the progression of colon cancer. However, NSAIDs have been effective against colon cancer cell lines that do not express COX-1 or COX-2 enzymes (8, 22) and also against mouse embryo fibroblasts that are null for both COX-1 and COX-2 genes (23). Furthermore, NSAID metabolites that do not appreciably affect the catalytic activity of COXs retain their anti-tumor properties in tissue culture (24–26) and animal experiments (13, 14). These data provide powerful evidence that COX is not the only target for NSAIDs in the colon. Other pharmacological targets for NSAIDs have been identified. Recently, He et al . (27) demonstrated that sulindac inhibits DNA binding of the PPARδ nuclear receptor, reversing the effects of adenomatous polyposis coli (APC) mutations in colorectal cancer cells. However, again this mechanism did not fully account for NSAID-induced growth inhibition of the cells studied, which indicates that other mechanisms may also be involved.The NF-κB signaling pathway was previously identified as a target for the anti-inflammatory effects of aspirin and related NSAIDs (28, 29). NF-κB generally exists as a heterodimer of the p50 and p65 polypeptides, bound in the cytoplasm by the inhibitor protein IκB (30, 31). Following cellular stimulation by a number of cytokines or pathogens, IκB is phosphorylated by the IκB kinase (IKK) complex at serines 32 and 36, then degraded by the 26S proteosome (30–33). Subsequently, NF-κB translocates to the nucleus, where it binds to regulatory elements within the promoter region of target genes.

In addition to regulating cellular responses to cytokines and pathogens, the NF-κB pathway plays an essential role in controlling cellular growth properties and apoptotic cell death (32, 33). Consistent with this, inappropriate signaling of NF-κB has been involved in tumor formation in lymphomas, breast, skin, and bladder cancers (34–37). Dejardin et al. (38) and others recently reported that colon cancer cell lines also show abnormally high NF-κB activity and low IκB levels (39, 40), which indicates that dysregulation of NF-κB may also contribute to colon carcinogenesis. In support of this notion, several growth regulatory genes that are known to be involved in colon cancer, including c-myc, p53, and COX-2, are regulated by NF-κB (34). Thus, the NF-κB signaling pathway is a strong potential target for the anti-tumor effects of NSAIDs in the colon.

Yamamoto et al. (41) previously demonstrated that NSAIDs inhibit cytokine-mediated IκB kinase (IKK) activation, and consequently NF-κB activation, in colorectal cancer cells and suggested that this specific interference is responsible for the antineoplastic activity of these

agents. However, in this and other studies showing NSAID-mediated inhibition of IKK, cells were pretreated with NSAIDs for a short period prior to a burst of tumor necrosis factor (TNF). Such experimental conditions are not representative of the *in vivo* situation in which colonic epithelium are continuously exposed to NSAIDs. Furthermore, in tissue culture experiments, NSAIDs inhibit the growth of colorectal cancer cells without additional cytokine stimulation (8–11). Therefore, the relevance of these studies to the chemopreventative effects of NSAIDs is unclear.

Our goal was to determine whether, in the absence of TNF or other stimulating agents, NSAIDs mediate an anti-tumor effect by targeting the NF-κB pathway. By performing these experiments without the addition of cytokines, we aimed to identify the underlying role of NF-κB in NSAID-mediated apoptosis. We demonstrate that, contrary to short-term treatment with aspirin plus cytokine, prolonged treatment with aspirin alone induces degradation of IκBα and nuclear translocation of NF-κB in colorectal cancer cells. Furthermore, NF-κB nuclear translocation is required for aspirin-induced apoptosis. These studies identify an intrinsic activity of aspirin that is highly relevant to colon cancer chemoprevention.

METHODS

Reagents

Aspirin and sodium salicylate (NaSaI) were supplied by Sigma (St. Louis, Mo.) and solubilized in water using 10 N NaOH, and the pH was adjusted to 7.0. All other chemicals and reagents were from standard commercial sources and of the highest quality.

Plasma salicylate

We investigated the relevance of the aspirin concentrations used in *in vitro* experiments. Patients were given 600 mg 4 times daily for 7 days. Blood was drawn at the end of this period, and plasma salicylate levels were measured by a standard colorimetric assay (Trinder method). Epidemiological studies have focused on populations that take NSAIDs for their analgesic effects, although there are no definitive data on the dose required for a chemopreventative effect. Hence, we studied subjects given a short course of analgesic doses of aspirin, because this seems to best reflect the use in the published cohort and case-control studies. Measurements of plasma salicylate levels in 10 study subjects ranged from 0.05 to 1.13 mM (mean 0.5 mM, SE +/- 0.14 mM), which is within the ranges used in the *in vitro* experiments.

Cell culture

SW480, HRT18 (genetically identical to DLD-1 and HCT-8/15) (42), HCT116, and CT26 colon cancer cell lines and the lung adenocarcinoma cell line, A549, are all available from the American/European Type Culture Collections (ATCC/ ECACC). SW480 cells were maintained in L-15 medium, HRT18 in RPMI 1640, HCT116 in McCoy's 5A medium, and CT26, A549, and 293 cells in DMEM. All media were supplied by Gibco BRL (Paisley, U.K.) and supplemented with penicillin (100 IU/ml), streptomycin (100 ug/ml), and 10% fetal calf serum (FCS).

Viability and apoptosis assays

Cells were plated at a density of 1×10^5 per 50-ml flask, grown until 60–80% confluent (generally 24–48 h), andthen treated continuously with 1–20 mM aspirin or sodium salicylate, in low-serum (0.5% FCS) medium, for a further 24 h, or as specified in text. Adherent cells were washed in PBS, harvested using a cell scraper, pipetted vigorously, and then counted on a hemocytometer. Nigrosin dye was added before counting to identify necrotic cells. Staining for cell surface phosphatidyleserine was used as a marker for apoptosis and was carried out using an Annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, Mass.) as per the manufacturer's instructions. Apoptotic cells were counted using fluorescent microscopy. For morphological analysis of apoptosis, adherent cells were fixed by acetic acid:ethanol (9:1 v/v) and mixed with acridine orange stain (0.5% final concentration) directly prior to analysis by fluorescent microscopy.

Immunoblot analysis and electrophoretic mobility shift assays

Cytoplasmic and nuclear extracts were made as previously described (43). Briefly, adherent cells were lysed for 5 min in lysis buffer (50 mM NaCl, 10 mM HEPES, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% Triton X-100, Complete protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany], 100 mM Pefabloc [Roche Diagnostics]), and then nuclei were pelleted by centrifugation at 6000 rpm for 15 min. Nuclei were lysed in hypotonic buffer (350 mM NaCl, 10 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, protease inhibitors, and Pefabloc as above) for 30 min, and debris was cleared by centrifugation at 13,000 rpm for 5 min. Bradford assays (BioRad, Hercules, Calif.) were used to measure protein content. Cytoplasmic extracts (30 μg) were resolved on 10% SDS polyacrylamide gels, and immunoblots were performed by standard procedures. The mouse monoclonal antibody to IκBα was a gift from Prof. R. Hay (St. Andrews). Membranes were probed with horseradish peroxidase-conjugated monoclonal antibody to Cu/Zn SOD (The Binding Site, Birmingham, U.K.) as a control for protein loading. The anti-pk-tag antibody was a gift from Dr. R. Randall (St. Andrews) and has been described (44). Antibody conjugates were detected using Amersham (Little Chalfont, U.K.) ECL protocol.

Electrophoretic mobility shift assays (EMSAs) were carried out by incubating nuclear extracts (6 μg) with binding reaction mix (1X binding buffer [50 mM KCl, 20 mM HEPES, 5% glycerol, 1 mM EDTA, 1 mM DTT], 1 μg BSA, 1 μg poly dI-dC, 25 fmol radioactively labeled oligo-DNA in a final volume of 20 μl) for 30 min prior to analyses on a 4% native polyacrylamide gel. For supershift assays, nuclear extracts were preincubated with 1 ul of anti-p50 or p65 antibodies (1 μg/μl) (Santa Cruz, Santa Cruz, Calif.) for 1 h on icebefore other assay reagents were added. Double-stranded oligonucleotides for wild-type and mutant NF-κB were obtained from Santa Cruz.

Immunocytochemical staining

SW480 cells were grown on sterilized coverslips until 60–80% confluent and then treated with aspirin in low-serum (0.5% FCS) medium for 24 h. Cells were washed twice in cold PBS for 10 min each and then fixed in 1:1 methanol:acetone at –20°C for 20 min before being blocked in 10% nonimmune donkey serum (Sigma) for a further 30 min. Negative control coverslips were

kept in 1.5% donkey serum without exposure to either of the primary antibodies, rabbit polyclonal to p65 or goat polyclonal to p50 (Santa Cruz) whereas the remaining coverslips were incubated for 1 h with the primary antibody at a 1:100 dilution in 1.5% donkey serum. After extensive washing in PBS, all coverslips were incubated with either FITC-conjugated antirabbit (for p65) or antigoat (for p50) (Jackson ImmunoResearch, West Grove, Penn.) for 30 min and then washed again in PBS. Coverslips were mounted in Vectastain (Vector Laboratories, Burlingame, Calif.) containing 1:25 xmg/ml DAPI, and cells were analyzed using fluorescent microscopy.

Transfections and generation of stable IκB$^{s32/36}$ expressing cell lines

The IκB$^{s32/36}$ plasmid was a gift from R. Hay and contains IκBα mutated at serines 32 and 36, cloned into pcDNA3 with a C-terminal pk-tag (43). For transient transfection experiments, cells were transfected using Lipofectamine (as described by the manufacturer, Gibco BRL), grown for 24 h in low-serum (0.5% FCS) medium, and then treated continuously with aspirin (0–10 mM) for another 24 h. Immunoblots were performed on cytoplasmic extracts using anti-pk tag antibody as described above. Transfected CT26 and HRT18 cells were grown under Geneticin (Gibco BRL) selection, and stable transfectants were screened by immunoblotting, using the anti-pk-tag antibody. Expressing clones were grown in the absence of Geneticin during aspirin treatment.

Aspirin treatment of clinical samples

Normal mucosa and tumor biopsies were collected from patients undergoing resection for colorectal cancer. Biopsies were washed in PBS, finely sliced, and incubated for 5 h in RPMI medium containing aspirin or carrier control. Tissue fragments were homogenized in lysis buffer, after which protein extraction and immunoblotting were performed, as described above.

RESULTS

Aspirin induces apoptosis in colorectal cancer cells

To establish that pharmacologically relevant doses of aspirin induce the death of colorectal cancer cells, we incubated SW480 cells with 1–10 mM aspirin for 24 h, or 0.5–2 mM aspirin for 48 h. These concentrations are comparable to salicylate levels that we measured in plasma (0.05–1.13 mM, mean 0.5 mM, SE +/- 0.14 mM, n=10) from patients given a short course of analgesic doses of aspirin (600 mg 4 times daily), which we considered to reasonably represent the use in published cohort and case-control studies of chemoprevention. We found that aspirin induced a dose-dependent reduction in the number of viable colorectal cancer cells (SW480) (Fig. 1A, B). This was accompanied by an increase in the percentage of cells showing externalization of phosphatidylserine (PS), a marker for apoptosis detected by FITC-annexinV binding (Fig. 1A, B). Treated cells also showed morphological features of apoptosis, including condensed cytoplasm, granular chromatin, and irregular condensed nuclei (Fig. 1C). However, nuclear fragmentation and classical apoptotic bodies were rarely seen.

We wished to definitively establish that aspirin-treated cells, which appeared to be apoptotic, were dying by an active process rather than simply by toxic necrosis. Hence, we examined the requirement for *de novo* protein synthesis, one of the original criteria used for identifying

apoptosis (45). Inhibition of protein synthesis by cyclohexamide (10 μM) abrogated the aspirin-induced (10 mM) growth inhibition (Fig. 1D), thereby confirming that aspirin-treated cells do indeed die by apoptosis.

Aspirin induces apoptosis in association with degradation of IκBα and nuclear translocation of NF-κB

To determine whether the apoptotic response we observed in SW480 cells was attributable to modulation of the NF-κB signaling pathway, we investigated the effects of aspirin on the NF-κB inhibitor protein, IκBα,using Western blot analysis. Prolonged (24–48 h) treatment with aspirin induced a dose-dependent reduction in cytoplasmic IκBα levels (Fig. 2A, B) that correlated directly with a reduction in the number of viable cells (Fig. 2C). Levels of control protein (Cu/ZnSOD) were unaffected by aspirin (data not shown). In the classical pathway of NF-κB activation, IκBα is phosphorylated at serines 32 and 36 before degradation by the 26S proteosome. To determine whether the aspirin-induced reduction in cytoplasmic IκBα was caused by phosphorylation and degradation of the protein, we first transfected SW480 cells with a dominant negative form of IκBα ($IκB^{S32/36}$-tag), which was resistant to phosphorylation at the critical S32/36 residues and hence resistant to signal-mediated degradation. The IκBα expression construct contained a C-terminal pk tag to allow discrimination between endogenous and recombinant protein (43). We found that aspirin (1–10 mM) had no effect on levels of the mutant protein (Fig. 3A), which indicates that these phosphorylation sites are important for the aspirin effect on IκBα. To examine the role of the proteosome in this effect, we preincubated SW480 cells with the MG132 proteosome inhibitor prior to treatment. This also blocked aspirin-induced reduction in IκBα levels (Fig. 3B). These results clearly indicate that prolonged treatment with aspirin mediates phosphorylation and proteosome-mediated degradation of IκBα. Furthermore, they also raise the possibility that IκBα degradation is associated with cell death.

IκBα degradation results in nuclear translocation of NF-κB complexes. To determine whether this also occurs after aspirin treatment, we used EMSAs to investigate nuclear levels of activated NF-κB. We found that aspirin-induced IκBα degradation was indeed accompanied by a dose-dependent increase in nuclear NF-κB DNA binding complexes (Fig. 4A, B). Binding of the induced NF-κB complex to the labeled probe was inhibited by competition with an excess of unlabeled wild-type, but not mutant, κB oligonucleotide (Fig. 4C). Supershift analysis with specific antibodies revealed the presence of p65 and, to a lesser extent p50, subunits of NF-κB in the induced complex (Fig. 4C). The findings from EMSAs were further corroborated by immunocytochemistry performed on aspirin-treated cells. Before aspirin treatment, p65 was mainly localized in the cytoplasm, but following 24 h of treatment with 10 mM aspirin there was a dramatic change, with extensive nuclear staining for this protein (Fig. 4D). Nuclear staining for p50 also increased following aspirin (10 mM) treatment, although this was less dramatic because of higher constitutive levels of nuclear p50 (data not shown). In contrast, there was no change in cellular localization of the control (Cu/ZnSOD) protein (data not shown). These results confirm that exposure of colorectal cancer cells to 24 h of aspirin activates the NF-κB pathway.

Kinetics of aspirin-induced IκBα degradation, NF-κB nuclear translocation, and cell death in colorectal cancer cells

To eliminate the possibility that NF-κB nuclear translocation was a consequence of cell death, we studied the kinetics of the aspirin effects on NF-κB signaling and cell viability. In contrast to the early transient IκBαα degradation observed following TNF-αα treatment, aspirin (10 mM) treatment induced complete degradation of IκBα after 2–5 h, and the protein remained absent for more than 24 h (Fig. 5A, 2A). Similarly, an increase in nuclear NF-κB DNA binding was observed 2 h after treatment and persisted for more than 16 h (Fig. 5B). In comparison, aspirin-induced apoptosis, determined by externalization of phosphatidylserine, was not detected until 16 h after treatment. These results show that apoptosis occurred after NF-κB nuclear translocation, and they raise the intriguing possibility that there is a causal relationship between aspirin-induced NF-κB activation and subsequent cell death.

Inhibition of NF-κB nuclear translocation inhibits aspirin-induced apoptosis

To definitively prove the relationship between NF-κB nuclear translocation and aspirin-mediated growth inhibition, we generated stable transfectants of HRT18 and CT26 colon cancer cells, which constitutively express the $I\kappa B^{S32/36}$-tag construct. Although we were able to study transient SW480 $I\kappa B^{S32/36}$ transfectants, numerous attempts at establishing stable $I\kappa B^{S32/36}$ transfectants of SW480 cells were unsuccessful. Clones expressing the transgene were identified by Western blot analysis using the anti-pk tag antibody. Two HRT18 ($I\kappa B^{S32/36}$h1, h28) and two CT26 ($I\kappa B^{S32/36}$ct3, ct4) clones were chosen, as these expressed particularly high levels of mutant protein (data not shown). As expected from our studies of transient transfected cells, in which we showed that $I\kappa B\alpha^{S32/36}$ is resistant to aspirin-induced degradation (Fig. 3A), nuclear translocation of NF-κB was inhibited in all independent clones expressing mutant IκBα when compared with respective parental cells. Parental lines showed substantial NF-κB nuclear translocation following aspirin treatment (1–5 mM) (Fig. 6A). If nuclear translocation of NF-κB were contributing to apoptosis, then inhibition should protect against aspirin-induced cell death. Indeed, the $I\kappa B^{S32/36}$ expressing clones grew in the presence of 1 mM aspirin compared with a 32.4% and 55.8% reduction in viable cell counts in parental HRT18 and CT26 cells, respectively (Fig 6B). Treatment with 5 mM aspirin also had significantly less effect ($P<0.05$) on the viability of mutant IκBα expressing clones than on the viability of parental lines (Fig. 6B). The decrease in viable cell number observed in parental cells was attributable to apoptosis, as shown by annexin V staining (data not shown). These data support the notion that aspirin-induced apoptosis of colorectal cancer cells depends on nuclear translocation of NF-κB, which is a consequence of phosphorylation and degradation of IκBα.

Cell specificity of aspirin-induced IκBα degradation and apoptosis

Epidemiological studies indicate that NSAID-mediated protection is relatively specific to colorectal tumors (46). Therefore, we wanted to determine whether cells not derived from colorectal tumors also responded to aspirin with similar effects on the NF-κB pathway and cell viability. In all 4 colorectal cancer cell lines tested (3 human and 1 mouse [CT26]), 5 mM aspirin induced substantial IκBα degradation and 10 mM aspirin induced almost complete degradation (Fig. 7A). In contrast, 10 mM aspirin had no effect on IκBα in embryonic kidney (293) or in lung adenocarcinoma (A549) cells (Fig. 7A). Substantial growth inhibitory effects were also observed in all colorectal cancer cell lines treated with 5 or 10 mM aspirin. Numbers of viable SW480, HRT18, CT26, and HCT116 cells decreased by 6.7-, 2.2-, 4.4-, and 2-fold, respectively,

following treatment with 5 mM aspirin (Fig. 7B). On the contrary, numbers of viable 293 cells increased in the presence of 5 mM aspirin, whereas A549 cells only showed a 1.2-fold decrease in viability following treatment (Fig. 7B). These results suggest that aspirin-mediated IκBα degradation and apoptosis may be cell-type-specific.

Aspirin induces IκBα degradation in normal colonic mucosa and tumors from rectal cancer patients

To investigate the potential clinical significance of our results, we treated biopsy samples of normal mucosa and tumor material from patients undergoing marginal resection for rectal cancer. The biopsies were maintained *ex vivo* as short-term explants and treated with 10 and 20 mM aspirin. IκBα degradation was observed in both the tumor and, to a lesser extent, in normal mucosa following 5 h treatment, whereas there was no effect on levels of the control (Cu/ZnSOD) protein (Fig. 8). These data confirm that clinical tumor biopsy material shows the same IκBα response observed in cell line experiments.

DISCUSSION

Our findings shed further light on the complex mode of action of NSAIDs. We show that, in the absence of additional NF-κB activating agents, aspirin induces IκBα degradation and nuclear translocation of NF-κB complexes in colorectal cancer cells. Furthermore, we provide compelling evidence that NF-κB nuclear translocation is predominantly responsible for aspirin-induced cell death. Taken together, our findings describe a novel mechanism of action for aspirin and have implications for the development of novel chemopreventative agents.

NSAIDs are known to reduce the growth of colorectal cancer cells both *in vitro* and *in vivo*, but the underlying cellular mechanism involved is still unclear. Some studies suggest NSAID-induced growth inhibition is due to cell cycle arrest (9, 11, 47), whereas others have reported induction of apoptosis and necrosis (7, 8, 11, 25, 48). . Here we demonstrate that aspirin induces two hallmarks of apoptosis in colorectal cancer cells: externalization of phosphatidylserine (Fig. 1A) and nuclear chromatin condensation (Fig. 1B). However, no nuclear fragmentation was evident by morphological analysis. This is consistent with data from Qiao et al. (10, 48), who also demonstrated that aspirin induces an "atypical" form of apoptosis that does not involve oligonucleosomal or nuclear fragmentation. *De novo* protein synthesis is required for apoptosis but not for passive necrosis (45). Our data showing that cyclohexamide inhibits aspirin-induced cell death confirms that this is an active process and indicates that the drug has specific antineoplastic activity that involves apoptosis.

Several pharmacological targets for NSAIDs have previously been identified, some of which are associated with an ability to inhibit the growth of colon cancer cells (28, 29, 49–52). For example, specific inhibition of COX-2 reduces adenocarcinoma formation in APC mutant mice (12, 53), and sulindac inhibits the DNA binding potential of PPARδ,thus inducing apoptosis of colon cancer cells (27). However, modulation of these targets does not fully account for the anti-tumor effects of these agents. The NF-κB transcription factor plays an important role in cell growth and death (9, 54), so we considered that this signaling pathway might be a candidate target for NSAIDs. We show that aspirin mediates a reduction in cytoplasmic IκBα levels in colorectal cancer cells that is time- and concentration-dependent (Figs. 2 and 5). This reduction

is blocked by pretreatment of cells with the MG132 proteosome inhibitor and by mutation of IκBα at the critical S32/36 phosphoacceptor sites (Fig. 3), which indictates that it is due to phosphorylation and proteosome-mediated degradation of the protein. Using EMSAs and immunocytochemistry, we demonstrate that IκBα degradation results in nuclear translocation of p50/p65 NF-κB complexes (Fig. 4), confirming aspirin stimulates the NF-κB pathway. A direct link between aspirin-induced stimulation of the NF-κB pathway and apoptosis was implied by the correlation between IκBα degradation and reduced cell viability (Figs. 2C and 7). Time course experiments indicated that the NF-κB response to aspirin occurred before detectable cell death (Fig. 4), suggesting a causal relationship. This was confirmed in cells that we engineered to continuously express a dominant negative mutant IκBα (IκBα $^{S32/36}$). These cells showed inhibition of both aspirin-induced NF-κB nuclear translocation and apoptosis, compared with their parental counterparts (Fig. 6). We considered that the lack of apoptotic response to aspirin in the mutant IκBα expressing clones was due to their slower rate of growth, compared with parental cells. However, this is unlikely because they grew at a similar rate to other colorectal cancer cell lines (SW480) in which aspirin induced substantial apoptosis. Thus, from these experiments we conclude that aspirin-induced apoptosis is due, at least in part, to nuclear translocation of NF-κB complexes.

This study is the first to show stimulation of the NF-κB pathway by aspirin. Our data appear to contradict previous *in vitro* studies indicating that aspirin and its metabolite, sodium salicylate, bind to and directly inhibit IKKβ, the kinase responsible for phosphorylating IκBα (29, 41). However, in these studies, direct inhibition of IKKβ by aspirin required IKK to be pre-activated by TNF or a similar stimulating agent. Furthermore, recent studies performed on intact cells suggest that direct inhibition of IKK activity by sodium salicylate does not occur in an *in vivo* setting (55). Therefore, the discrepancy between our findings, using intact cells treated only with aspirin, and these other studies is most likely explained by differences in the experimental systems used. It is now of considerable interest to identify the upstream targets of aspirin responsible for stimulation of the NF-κB pathway. One potential candidate is the p38 MAP kinase, which is activated by sodium salicylate to induce apoptosis (56–58) and can activate the NF-κB pathway upstream of IκB phosphorylation (59). Alternatively, aspirin may indirectly activate NF-κB by inducing the secretion of soluble factors. Indeed, this might explain the delayed time course of aspirin-induced IκBα degradation we observe (Fig. 5). Identification of the upstream pathways leading to NF-κB activation would allow the development of agents designed specifically to target the proteins involved. Such agents would have potential as more potent and specific chemopreventative drugs.

Our finding that NF-κB nuclear translocation induces apoptosis in colorectal cancer cells is intriguing given that we (60) and others (40, 61, 62) have demonstrated that TNF-induced NF-κBis anti-apoptotic in colorectal cancer cells. The DNA binding specificity and selective transcriptional activation of downstream NF-κB target genes is determined by the dimeric composition of the NF-κB complex and by the transcriptional co-factors recruited to the complex (63). It seems likely that these are dependent on the kinetics of induction. We observed fundamental differences in the kinetics of IκBα degradation induced by aspirin compared with TNF (Fig. 5A). IκBα degradation was evident 2–5 h following aspirin treatment versus 30 min following TNF treatment, and aspirin-induced IκBα degradation persisted for more than 24 h, whereas TNF-induced degradation was transient. Therefore, the disparate cellular consequences of NF-κB activation induced by TNF and by aspirin may be accounted for by the differences in

kinetics of activation resulting in different NF-κB DNA binding complexes. Consistent with this hypothesis, we found predominantly p50 and p65 proteins in aspirin-induced NF-κB complexes (Fig. 4C) whereas a previous study from this laboratory reported predominantly c-rel/p65 complexes after TNF treatment in the same cell type (12). It was previously reported that NF-κB can be both pro- and anti- apoptotic in the same cell type, depending on the inducer, which provides additional support for this notion (33). Further work is under way in our laboratory to determine the composition of the aspirin-induced NF-κB DNA binding complexes and the downstream genes activated by these complexes. Using EMSAs and immunocytochemistry, we did not observe the high constitutive levels of nuclear p50/p65 NF-κB complexes that have been reported in other studies (38). However, we have some evidence that there may be subtle differences in levels of IκBα in patient colonic tumor material, compared with normal mucosa, which is now the subject of further detailed study.

Our finding that aspirin-induced IκBα degradation and apoptosis are more apparent in cells of colonic origin is in keeping with epidemiological studies indicating that NSAIDs impart relatively selective protection against colorectal cancer in humans. It seems unlikely that cellular specificity is related to overexpression of COX-2, because we observed aspirin-induced IκBα degradation in a cell line that does not express COX-2 (HCT-116) (52). Furthermore, salicylate, which is a very poor inhibitor of COX-2 (46), induced the same effect on colon cancer cells that we observed for aspirin (data not shown). That we detected some IκBα degradation in normal colonic mucosa, but substantially more in colonic tumor tissue, would suggest that this specificity is related to a tissue-specific protein that is dysregulated during carcinogenesis. Identification of molecular mechanisms underlying this specificity could provide surrogate markers that might indicate a likely NSAID response and so might inform clinical trials.

We observed activation of the NF-κB pathway and cell death at doses as low as 0.5 mM aspirin. This is comparable to the salicylate levels we measured in the plasma of human subjects (0.5 mM) and also those used in the treatment of arthritic disease (1–3 mM) (64). The doses of aspirin required for chemoprevention have not yet been fully established. Although some epidemiological data indicate that aspirin is chemopreventative when taken at low dose, it has been demonstrated that aspirin has unusual accumulation characteristics, in that serum levels increase disproportionately with dose following repetitive administration (65). Therefore, the lower doses used in this study may be comparable to levels achievable in a chemopreventative setting. Even at the highest doses studied, we show aspirin-induced cell death is an active process (Fig. 1C), indicating the mechanisms identified using these doses are significant to the antineoplastic activity of this agent.

In summary, the experimental data presented here show that aspirin induces degradation of IκBα and consequently, nuclear translocation of p50/p65 NF-κBcomplexes in colon cancer cells. This nuclear translocation is predominantly responsible for the ability of aspirin to induce apoptosis. The NF-κB and death response to aspirin appear relatively cell-specific since they were evident in numerous colon cancer cell lines but not in cell lines of noncolonic origin. Finally, aspirin induced stimulation of the NF-κB pathway in explants of normal and tumor tissue from colon cancer patients, a result that underscores the relevance of these molecular findings to clinical material. Defining the key elements of the anti-tumor effect described here will undoubtedly help inform the development of novel chemopreventative agents.

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Fig. 1

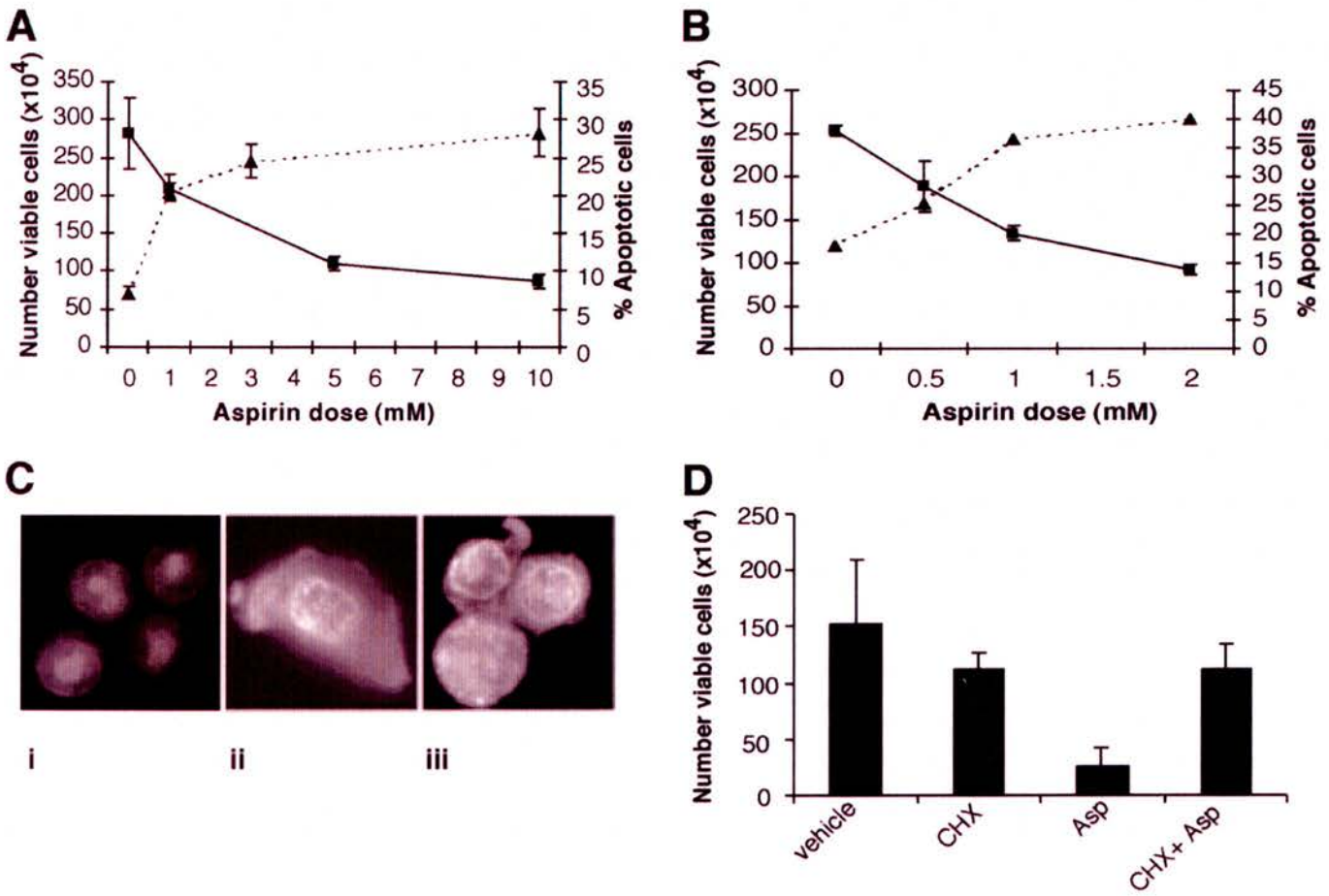

Figure 1. Aspirin induces apoptosis in colorectal cancer cells. **(A, B)** SW480 cells were treated with **(A)** 0—10 mM aspirin for 24 h or **(B)** 0—2 mM aspirin for 48 h in low-serum medium. The number of viable cells (■) was determined by hemocytometric counts. The percentage of apoptotic cells (▲) was determined by phosphatidylserine exposure, measured in a minimum of 200 cells using FITC-annexinV binding. The data presented represent the means of at least three independent experiments (+/- SE). A substantial proportion of aspirin-treated cells showed phosphatidylserine exposure, which is indicative of membrane unpacking and apoptosis. **(C)** Fluorescent microscope images (× 40) of acridine orange-stained SW480 cells, treated for 24 h with buffer **(i)** or 5 mM aspirin **(ii** and **iii)**. Aspirin-treated cells have morphological features of apoptosis. **(D)** Cyclohexamide (10 μM) (CHX) blocks aspirin-induced growth inhibition of SW480 cells. Hemocytometric counts of viable SW480 cells following 24 h of treatment with 10 mM aspirin in the presence or absence of CHX.

Fig. 2

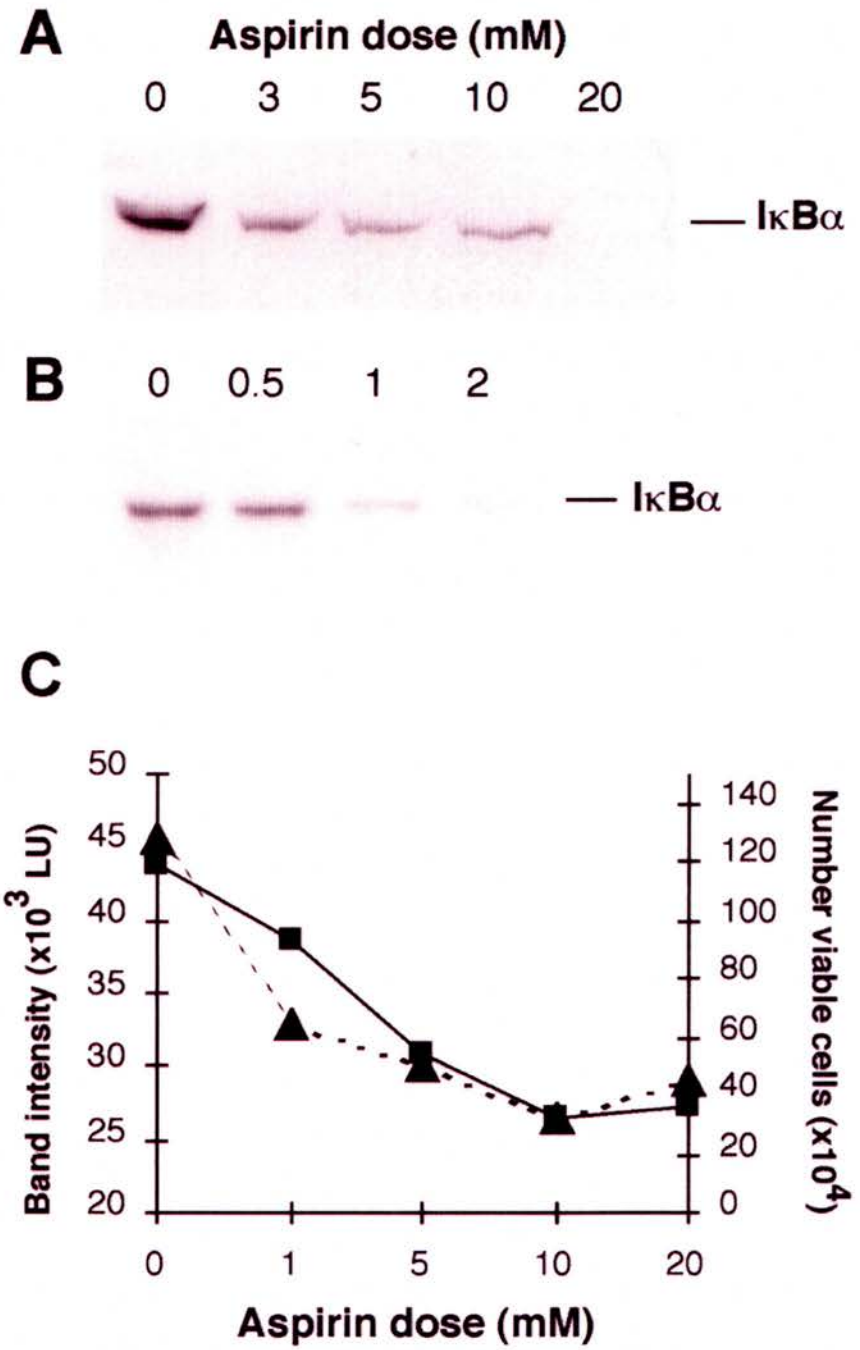

Figure 2. Aspirinmediates a reduction in cytoplasmic IκBα levels in colorectal cancer cells. **(A, B)** Anti-IκBα Western blots of cytoplasmic extracts from SW480 cells treated with **(A)** 0—20 mM aspirin for 24 h or **(B)** 0–2 mM aspirin for 48 h. **(C)** Reduction in IκBα levels is concomitant with aspirin-induced apoptosis. IκBα protein levels (▲) were quantified from Western blots using standard software (given as light units,LU) and correlated with viable cell number (■). IκBα levels are corrected for differences in protein loading between tracks using Cu/ZnSOD levels. One representative experiment is shown.

Fig. 3

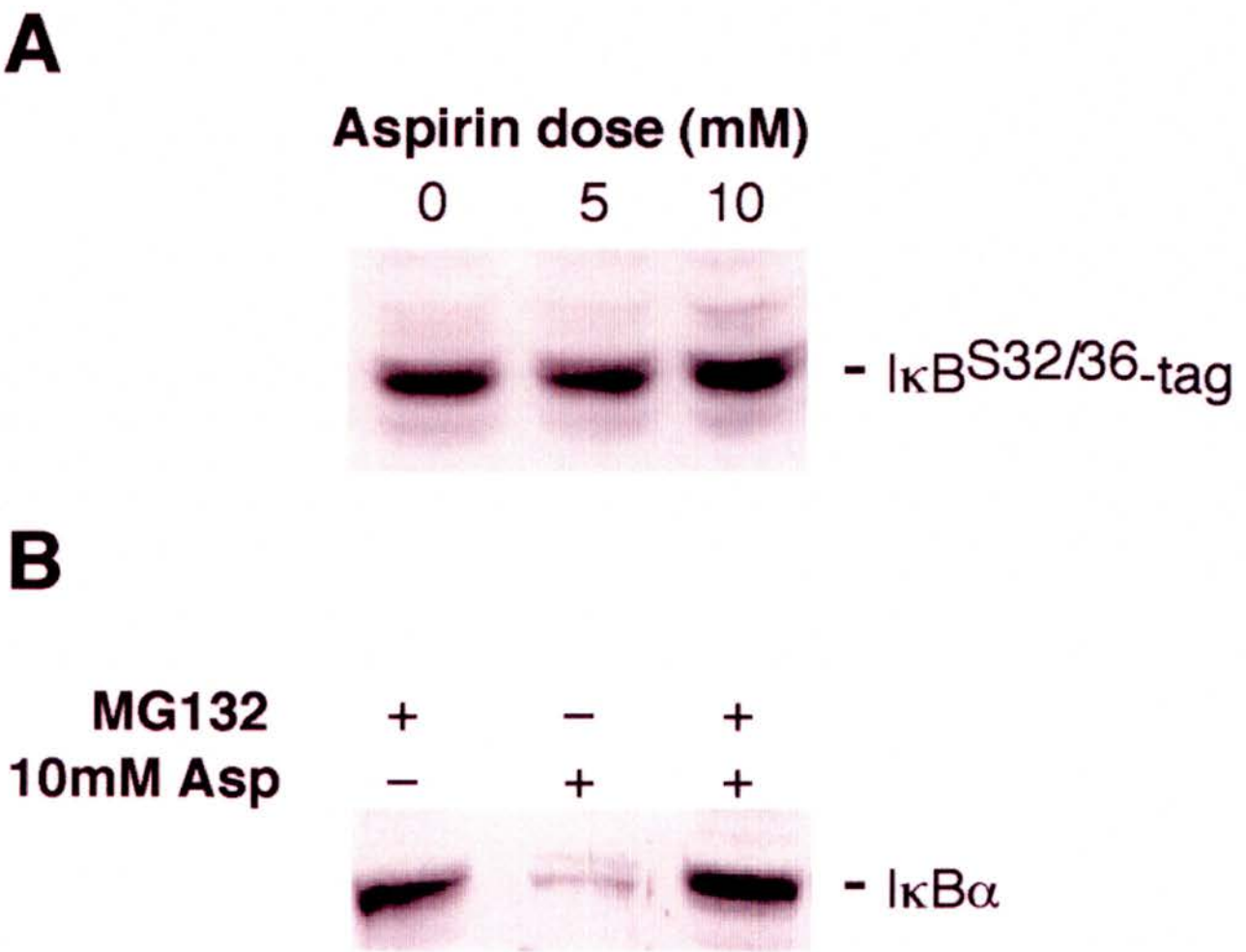

Figure 3. Aspirin induces phosphorylation and proteosome-mediated degradation of IκBα. **(A)** IκBα mutated at the ser-32 and ser-36 phosphorylation sites is unaffected by aspirin. Anti-pk tag Western blot of cytoplasmic extracts from HW480 cells transfected with an $I\kappa B^{S32/36}$-pk tag expression vector, prior to 24 h of treatment with 0-10 mM aspirin. **(B)** Proteosome inhibition blocks aspirin-mediated reduction in cytoplasmic IκBα. Anti-IκBα Western blot of cytoplasmic extracts from cells incubated with the MG132 proteosome inhibitor (50 μM) for 5 h, prior to aspirin (10 mM) treatment for 24 h.

Fig. 4

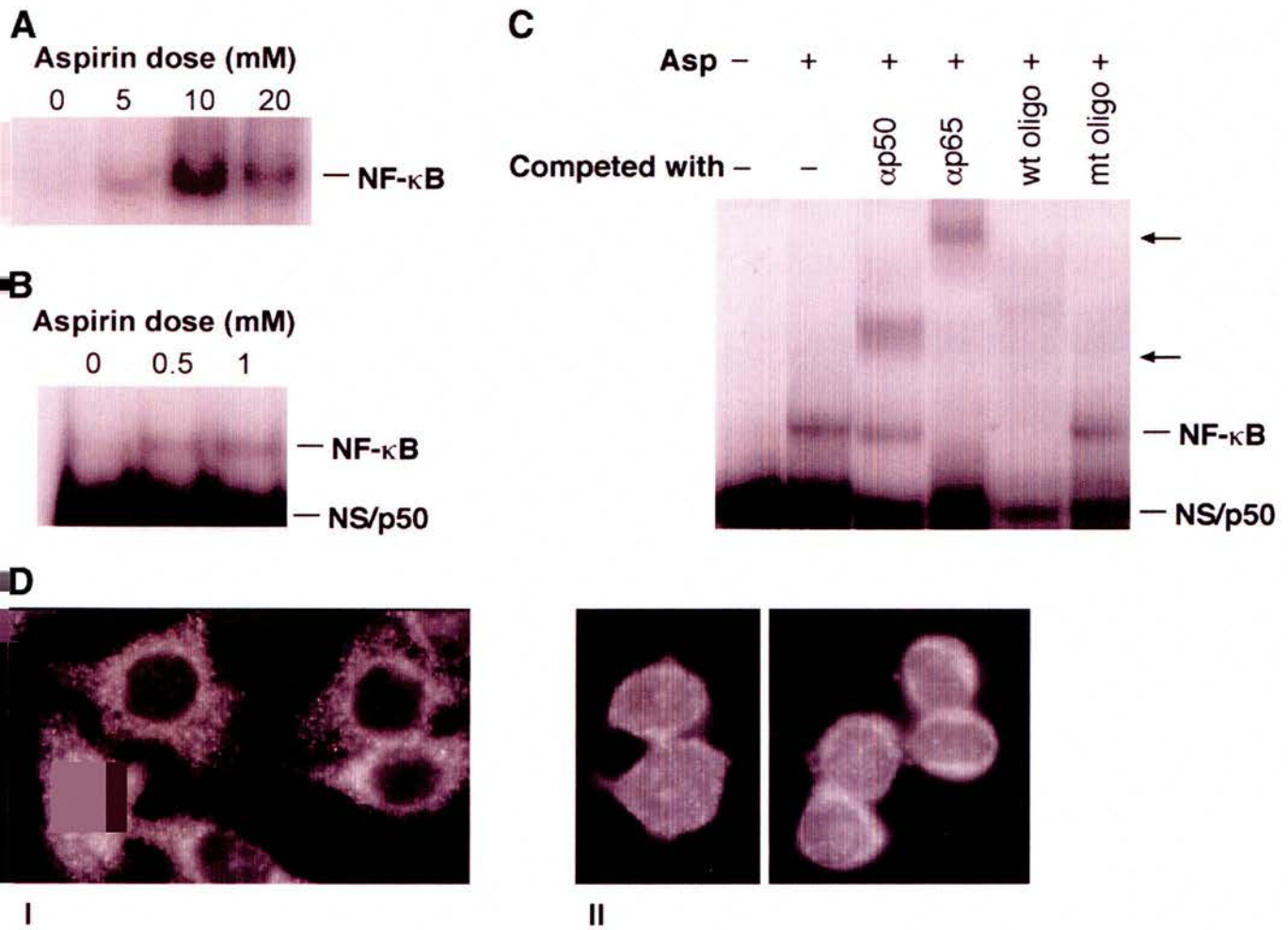

Figure 4. Aspirin induces nuclear translocation of NF-κB. **(A, B)** Electrophoretic mobility shift assays using NF-κB consensus oligonucleotide, performed on nuclear extracts from SW480 cells treated with **(A)** 0–20 mM aspirin for 24 h or **(B)** 0–1 mM aspirin for 48 h. **(C)** Competition electrophoretic mobility shift assays indicated the identity and specificity of NF-κB-induced complexes. The band induced by 10 mM aspirin was supershifted (indicated by arrows) when antibodies to αp50 and αp65 were included in the assay. Excess (100-fold) unlabeled wild-type (wt) NF-κB oligonucleotide competed with the binding of p50/p65 heterodimers to the labeled probe but not excess (100-fold) mutated (mt) NF-κB sequences. Supershift and competition assays indicate the lower band is a combination of nonspecific (NS) binding and p50 homodimers. **(D)** Micrographs (63 ×) showing immunofluorescent analysis of SW480 cells either untreated (I) or treated with 10 mM aspirin for 24 h (II), then immunocytochemically stained with p65 antibodies. The signal was detected using FITC-conjugated secondary antibody.

Fig. 5

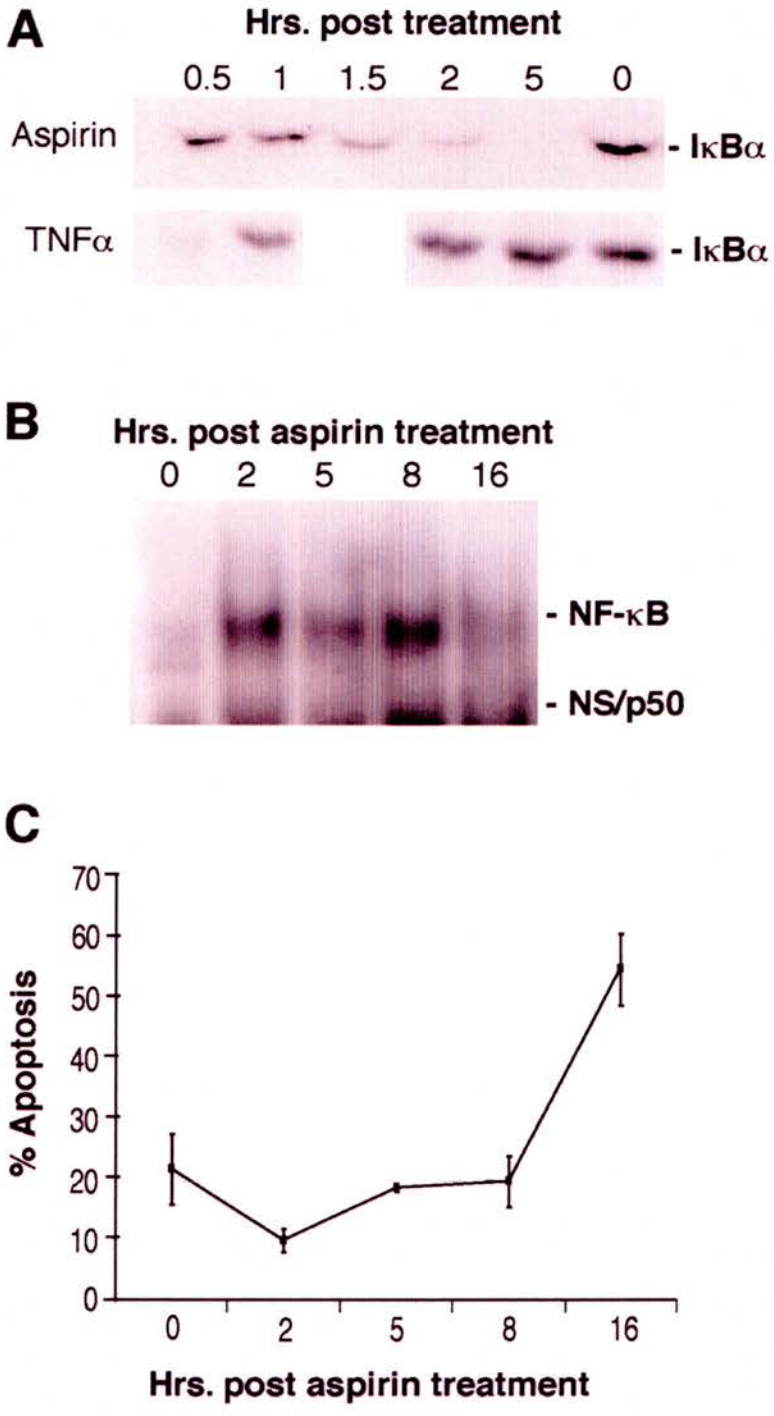

Figure 5. Kinetics of aspirin-induced NF-κB activation and cell death. **(A)** Western blot of cytoplasmic extracts from SW480 cells treated with 10 mM aspirin (upper panel) or 100 ng/ml TNFα (lower panel) for 0—5 h. Anti-IκBα antibody was used. **(B)** Electrophoretic mobility shift assays using NF-κB consensus oligonucleotide, performed on nuclear extracts from SW480 cells treated with 10 mM aspirin for 0—16 h. Induction of NF-κB is apparent after 2 h. **(C)** SW480 cells were treated with 10 mM aspirin for 0–16 h; then the percentage of apoptotic cells was determined in a minimum of 200 cells by annexinV-FITC binding to externalized phosphatidylserine. The data shownn represent the means of at least three independent experiments (+/- SE).

Fig. 6

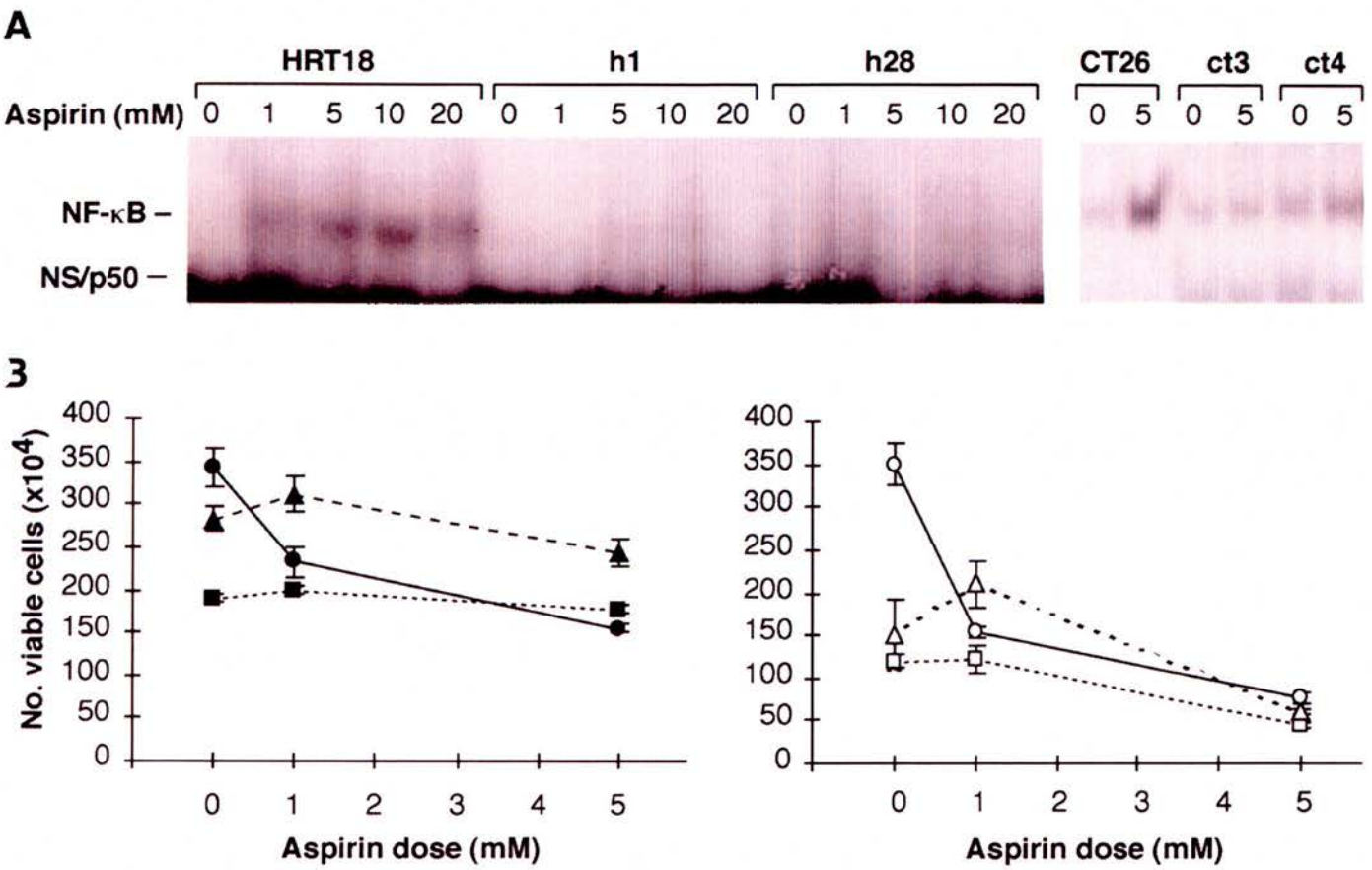

Figure 6. IκB$^{s32/36}$ inhibits aspirin-mediated nuclear translocation of NF-κB and apoptosis. HRT18 (h) and CT26 (ct) colorectal cancer cells were transfected with the IκB$^{s32/36}$-tag plasmid, and stable clones were generated. Antitag immunoblots identified 2 clones from each parental cell line that showed high levels of expression of the mutant protein (IκB$^{s32/36}$ h1, h28, ct3, and ct4) (not shown). **(A)** EMSAs, performed on nuclear extracts from HRT18 and CT26 parental cells, and IκB$^{s32/36}$ h1, h28, ct3, and ct4 clones, treated with aspirin for 24 h. Aspirin-induced p50/p65 complexes are not present in mutant IκBα expressing cells. **(B)** Hemocytometric counts of viable HRT18 (●) and CT26 (○) parental cells and IκB$^{s32/36}$ h1 (■), h28 (▲), ct3 (□), and ct4 (△) clones following 24 h of aspirin treatment. Data presented represent the mean of 4 independent experiments (+/- SE). At 1 and 5 mM aspirin, the difference between the number of viable treated and nontreated cells was significantly less in the clones expressing the mutant protein (P<0.05) compared with the parental cells.

Fig. 7

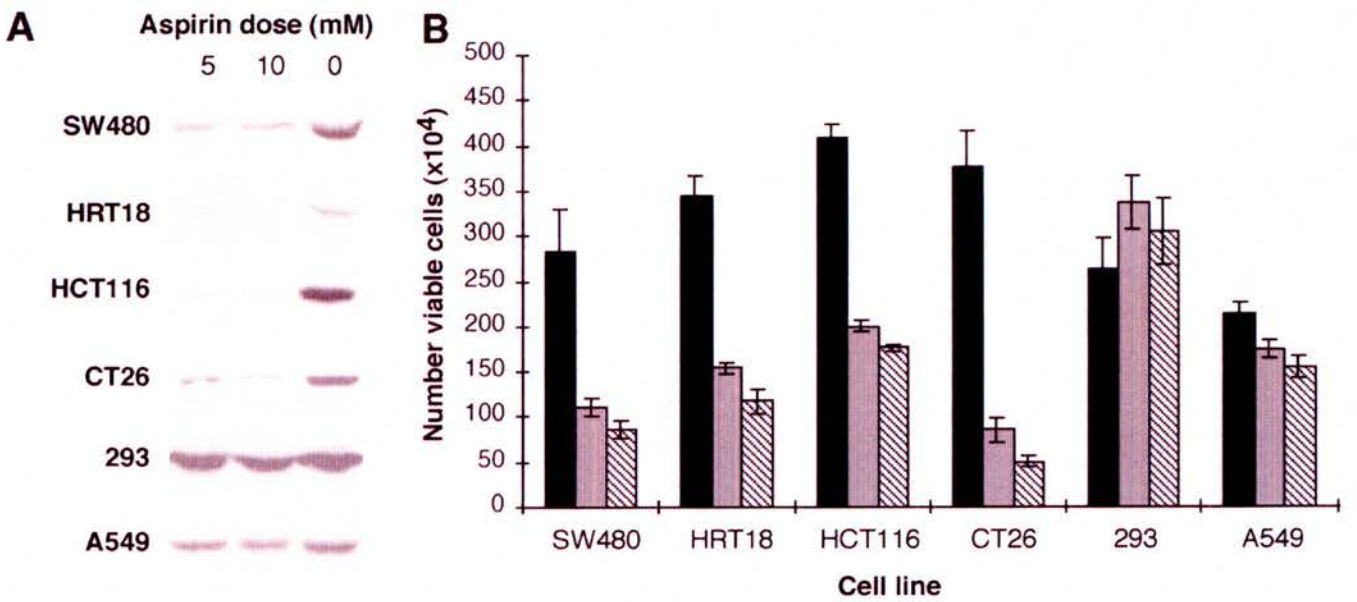

Figure 7. Cell specificity of aspirin-mediated IκBα degradation and growth inhibition. SW480, HRT18, HCT116, and CT26 (mouse) colon cancer cells and 293 (embryonic kidney) and A549 (lung adenocarcinoma) control cells were treated with aspirin (0—10 mM) for 24 h. **(A)** Anti-IκBα immunoblot of cytoplasmic extracts. **(B)** Hemocytometric counts of viable cells (mean of four experiments, +/- SE).

Fig. 8

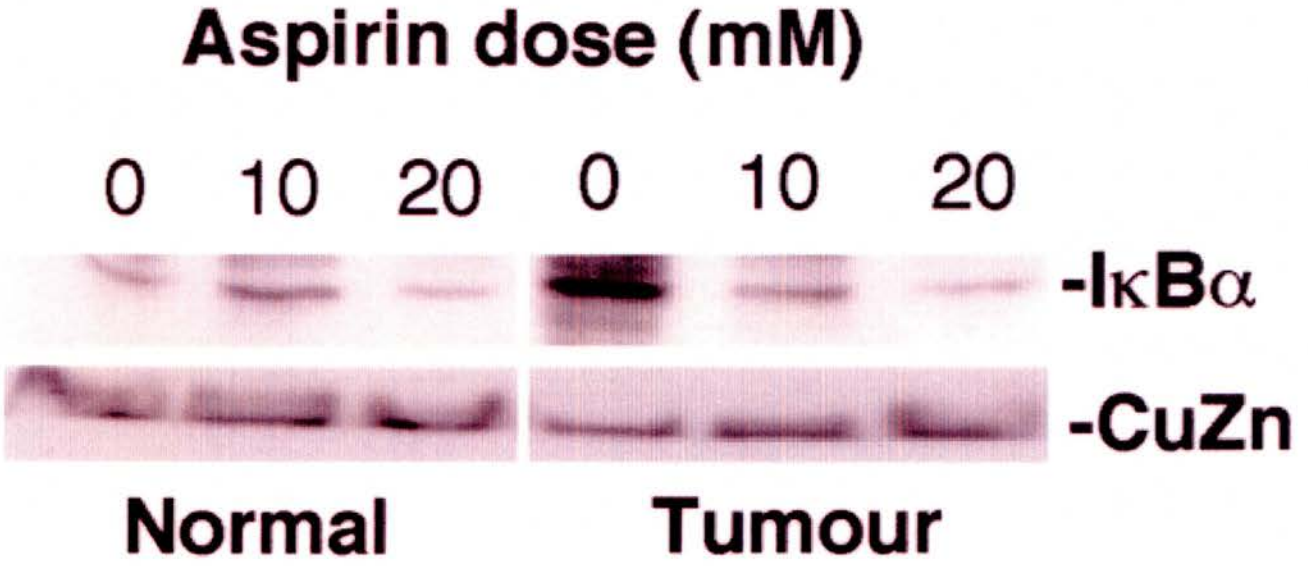

Figure 8. Aspirin induces IκBα degradation *ex vivo*. Anti-IκBα and Cu/ZnSOD (control) immunoblots of cytoplasmic extracts from clinical biopsy material from rectal tumors and normal colonic mucosa, treated in tissue culture for 5 h with 0-20 mM aspirin.

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Evidence for colorectal cancer cell specificity of aspirin effects on NFκB signalling and apoptosis

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Epidemiological evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) protect against colorectal cancer (CRC) to a greater degree than other non-gastrointestinal cancers, but the molecular basis for this difference is unknown. We previously reported that aspirin induces signal-specific IκBα degradation followed by NFκB nuclear translocation in CRC cells, and that this mechanism contributes substantially to aspirin-induced apoptosis. Here, we explored the hypothesis that cell-type specific effects on NFκB signalling are responsible for the observed differences in protection by aspirin against CRC compared to breast and gynaecological cancers. We also assessed whether COX-2 expression, mutation status of adenomatous polyposis coli (APC), β-catenin, p53, or DNA mismatch repair (MMR) genes in CRC lines influenced aspirin-induced effects. We found that aspirin induced concentration-dependent IκBα degradation, NFκB nuclear translocation and apoptosis in all CRC lines studied. However, there was no such effect on the other cancer cell types, indicating a considerable degree of cell-type specificity. The lack of effect on NFκB signalling, paralleled by absence of an apoptotic response to aspirin in non-CRC lines, strongly suggests a molecular rationale for the particular protective effect of NSAIDs against CRC. Effects on NFκB and apoptosis were observed irrespective of COX-2 expression, or mutation status in APC, β-catenin, p53 and DNA MMR genes, underscoring the generality of the aspirin effect on NFκB in CRC cells. These findings raise the possibility of cell-type specific targets for the development of novel chemopreventative agents.
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Keywords: IκB; NFκB; NSAIDs; chemoprevention; colorectal cancer

Colorectal cancer (CRC) is common in developed countries (Parkin *et al*, 1999) and is a major contributor to cancer-related morbidity and mortality. Chemoprevention is an inherently appealing approach to combat the disease, and non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with a substantial reduction in CRC incidence and mortality (Thun *et al*, 1993; Collet *et al*, 1999; Langman *et al*, 2000). Combined case–control data, including over 30 000 CRC cases, indicate a 45% reduction in the risk of developing CRC in subjects taking NSAIDs. Although there is evidence for a protective effect of NSAIDs against non-gastrointestinal cancers, the data are less convincing and the risk reduction much less. In breast cancer, reports show conflicting results and a recent meta-analysis revealed a risk reduction of only 13% in case–control studies (Khuder and Mutgi, 2001), considerably lesser than that in CRC. Similarly, in endometrial and ovarian cancer, the available evidence suggests that NSAIDs confer little, if any, protection (Cramer *et al*, 1998; Rosenberg *et al*, 2000; Fairfield *et al*, 2002; Meier *et al*, 2002). Collectively, published data suggest that there is considerable heterogeneity of NSAID anti-tumour effect *between* cancer types. The particular protective effect against CRC suggests the possibility that aspirin might target distinct molecular pathways in colonic epithelial cells. Elucidation of the molecular mechanism of this apparent differential sensitivity would lend further insight into both the mode of action of NSAIDs as well as identification of molecular markers of response.

The anti-tumour activity of NSAIDs has primarily been attributed to inhibition of the cyclooxygenase-2 enzyme (COX-2) and the resultant decrease in production of prostaglandins, as this remains the best-characterised effect (Vane, 1971). However, accumulating evidence from animal and cell culture experiments has shown that COX-2 inhibition is not the sole basis of NSAID anti-tumour activity (Alberts *et al*, 1995; Hanif *et al*, 1996; Elder *et al*, 1997; Piazza *et al*, 1997), suggesting that other targets are also involved. We previously reported that aspirin activates the NFκB signalling pathway and that this mechanism is of central importance to aspirin-mediated apoptosis in CRC cells (Stark *et al*, 2001). The NFκB transcription factor is normally sequestered in the cytoplasm by an inhibitor protein, IκBα. Following stimulation of the NFκB pathway, IκBα is phosphorylated, ubiquitinated and targeted for proteosomal degradation. Dissociation from IκBα results in translocation of NFκB to the nucleus, where it contributes to the co-ordinated transcription of genes involved in inflammation, cell proliferation and apoptosis (Pahl, 1999). Our previous work demonstrated that aspirin induces time- and dose-dependent signal-specific degradation of IκBα, nuclear translocation of NFκB and apoptosis in CRC cells. Time-course experiments indicated that IκBα degradation and NFκB nuclear

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translocation preceded cell death, suggesting a causal relationship. This was confirmed in cells we engineered to continuously express a dominant-negative mutant IκBα (IκBαS32/36), which showed inhibition of both aspirin-induced NFκB nuclear translocation and apoptosis compared to their parental counterparts (Stark *et al*, 2001). This work alluded to the notion of specificity since the NFκB response was not observed in the control cell lines 293 HEK and A549, which were non-colorectal in origin.

Here, we focus on the important issue of the specificity of aspirin's protective effects, as observed in epidemiological studies, and we set out to determine whether cell-type specific effects on the NFκB signalling pathway reflect the differential protective effects of aspirin in different cancer types. In particular, we wished to determine whether the lower protective effect observed for breast, ovarian and endometrial cancer can be explained by differing effects on the NFκB signalling pathway. We also investigated the generality of the NFκB response to aspirin in CRC by studying a panel of CRC cell lines with different genetic defects common in bowel malignancy. Here, we present evidence showing clear differences in NFκB response that parallel the epidemiological data, supporting the notion that the ability of aspirin to modulate the NFκB signalling pathway is a key determinant of the anti-tumour effect and that this is cell-type specific. Our findings provide further insight into the complex mechanisms by which NSAIDs exert an anti-tumour effect in CRC cells, and raise the possibility of cell-type specific molecular targets in CRC.

MATERIALS AND METHODS

Cell line culture and treatment

The CRC cell lines used were HRT-18, SW480, HT-29, DLD-1, LoVo and HCT116; breast cancer lines were T47-D, MCF-7 and MDA-MB-231; ovarian cancer line was A2780 and endometrial cancer line was HEC-1-A. All cancer cell lines are available from the American Type Culture Collection. The mutation status for the adenomatous polyposis coli (APC), p53, β-catenin and DNA mismatch repair (MMR) genes of the cell lines studied is shown in Table 1. Cell lines were grown as monolayers (37°C in 5% CO_2) in RPMI (HRT-18, DLD-1 and A2780), DMEM (HT-29, T47-D, MCF-7, MDA-MB-231, HEC-1-A), L-15 (SW480) and McCoy's 5A media (HCT116) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (media supplied by Gibco BRL, Paisley, UK). Cells were plated (1×10^6 cells/50 ml flask) and grown until 60–70% confluent, prior to treatment with aspirin or carrier control at the same concentrations as the aspirin treatment. Aspirin (Sigma, St Louis, USA) was prepared as a 0.5 M stock solution in distilled water (final pH 7.0). Growth medium was replaced with the respective low serum (0.5% FCS) medium and cells were treated with aspirin at 1, 3, 5 and 10 mM for 24 h (or 72 h), or with carrier as a control.

Cell viability and determination of apoptosis

Adherent cells were harvested and viable cell number determined by haemocytometric counts with nigrosin exclusion. IC_{50} values for the CRC cell lines were calculated using the XL*fit* 3™ software. Apoptosis was detected via its interaction with annexin V using an Annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA, USA), as per the manufacturer's instructions. Briefly, the medium from the flask of adherent cells was transferred to a conical tube on ice to harvest any floating cells. Cells were then washed with 2 ml of PBS, which was also added to the tube to collect any cells dislodged during washing. Cells were incubated with 1 ml of trypsin : versene (volume per volume) just until the cells detached and then resuspended in the conical tube containing the media with the floating and washed cells. Cells were counted using a haemocytometer and resuspended in cold 1 × binding buffer to approximately 1×10^6 cells ml^{-1}. Media-binding reagent (10 μl) was added to 0.5 ml of the cell suspension, which was incubated with 1.25 μl of annexin V-FITC for 15 min at room temperature in the dark. Annexin V was then removed by centrifugation at 1000 *g* for 5 min and the cells were resuspended in 0.5 ml of cold 1 × binding buffer and placed on ice. The counting was done using a haemocytometer (two counting grids) in duplicate and this was carried out immediately following staining of cells, as apoptosis is an ongoing process and the FITC signal may be lost after an hour.

Table 1 Mutation status of cancer cell lines studied

Cell line	APC	β-catenin	p53	MMR
HRT-18	Mutant	Wild type	Mutant	Deficient
SW480	Mutant	Wild type	Mutant	Proficient
HT-29	Mutant	Wild type	Mutant	Proficient
DLD-1	Mutant	Wild type	Mutant	Deficient
LoVo	Mutant	Wild type	Wild type	Deficient
HCT-116	Wild type	Mutant	Wild type	Deficient
MCF-7	Wild type	Wild type	Wild type	Proficient
MDA-MB231	Wild type	Wild type	Mutant	Not known
T47D	Wild type	Wild type	Mutant	Proficient
A2780	Wild type	Wild type	Wild type	Proficient
HEC-1-A	Not known	Not known	Not known	Deficient

Western blotting

Cells were washed with PBS, centrifuged (1200 r.p.m., 10 min) and cell pellets resuspended in lysis buffer (50 mM NaCl, 10 mM HEPES, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% Triton X-100) containing complete Protease Inhibitor Cocktail and 100 mM Pefabloc (Roche Diagnostics, Manheim, Germany). The cell suspension was centrifuged (6000 r.p.m., 15 min, 4°C) and the supernatant containing cytoplasmic proteins aliquoted. Protein content was measured by the method of Bradford (BioRad, Hercules, California, USA). Cytoplasmic proteins (30 μg) were separated on a 10% SDS–PAGE gel, transferred to a polyvinylidine difluoride membrane (BioRad) and blocked in 4% non-fat dry milk solution with 0.3% Tween20 (Sigma). Membranes were probed with a sheep polyclonal IκBα antibody (a gift from Professor R Hay, University of St Andrews, UK), rabbit polyclonal p65 antibody (Santa Cruz, California, USA) or mouse monoclonal COX-2 antibody (Cayman Chemicals, Michigan, USA). COX-2 electrophoresis standard (Cayman Chemicals) was used to indicate the correct COX-2 band. Monoclonal antibody to Cu/Zn SOD (The Binding Site, Birmingham, UK) and to actin (Santa Cruz) was used as a control for protein loading. Antigen–antibody complexes were visualised with chemiluminescence (Amersham ECL Reagents, UK).

Immunofluorescence analysis

Cells grown to 60–70% confluence on glass coverslips were treated with carrier or 10 mM aspirin for 24 h (in the respective 0.5% FCS medium). After treatment, cells were washed with PBS, fixed with acetone : methanol (volume per volume) (−20°C, 10 min) and blocked in 10% pre-immune donkey serum (Sigma) for 1 h. Rabbit polyclonal antibody to NFκB p65 (Santa Cruz) was applied for 1 h, followed by incubation with FITC-conjugated donkey anti-rabbit IgG for 1 h. The nuclei were stained with DAPI and the coverslips mounted with Vectashield (Vector Laboratories, Burlingame, California, USA).

RESULTS

Colorectal cancer cells are more susceptible to aspirin-induced apoptosis than non-CRC cells

We studied the effect of aspirin on the growth of a panel of CRC cell lines (HRT-18, SW480, HT-29, DLD-1, LoVo and HCT116) in comparison to cell lines derived from other cancer types: breast (MCF-7, MDA-MB-231, T47D), ovarian (A2780) and endometrial (HEC-1-A). The non-CRC cell lines were chosen based on epidemiological data, where there is some evidence to suggest a protective effect in breast cancer and less so in ovarian and endometrial cancer.

In triplicate dose–response experiments, cell lines were treated for 24 h with aspirin at concentrations of 1, 3, 5 and 10 mM, and viable cell number determined by haemocytometric counts. We found a concentration-dependent decrease in viable cell number in each of the six CRC cell lines studied (Figure 1A). In contrast, there was no demonstrable effect of aspirin on the viability of the non-CRC cell lines MCF-7, MDA-MB-231, A2780 and HEC-1-A (Figure 1A). Interestingly, the T47D breast cancer cells did exhibit a dose-dependent reduction in viability, although this effect was not so pronounced as that seen in CRC cells at low aspirin concentrations. The IC_{50} values were calculated from the growth curves of the aspirin-treated CRC cell lines only, as there was no consistent reduction in cell viability in the non-CRC cell lines (Table 2). The mean IC_{50} value for the CRC cell lines was 2.38 mM and the greatest incremental reduction in viability in these cells was observed between 0 and 1 mM concentrations, which is comparable to serum concentrations attainable in humans (Pachman *et al*, 1979).

We next wished to establish whether the reduction in viable cell number that we observed in the CRC cell lines was due to induction of apoptosis. Annexin-V binding of phosphatidylserine residues externalised during apoptosis was used to determine the proportion of cells undergoing programmed cell death in response to increasing concentrations of aspirin. We found that aspirin treatment induced a concentration-dependent increase in apoptosis in all six of the CRC cell lines studied, confirming that induction of apoptosis is responsible for the observed reduction in cell viability (Figure 1B). There was no dose-dependent increase in apoptosis in the non-CRC cell lines following aspirin treatment, which was consistent with the lack of effect on cell viability (Figure 1A). Although the T47D breast cancer cells did exhibit a reduction in viable cell count, this effect was less marked than that seen in CRC cells and, furthermore, there was no increase in apoptosis in this cell line. To confirm that the non-CRC cells were less sensitive to apoptosis, we treated three non-CRC cell lines (two breast and one ovarian) and one CRC cell line (SW480) with aspirin for a longer time period of 72 h. Indeed, the non-CRC cell lines were far less susceptible to apoptosis compared to the CRC cell line despite treatment with aspirin for 72 h (Figure 1C). These findings demonstrate that the anti-tumour activity of aspirin has a substantial degree of specificity for CRC cells *in vitro*, reflecting the epidemiological evidence for a greater protective effect against CRC compared to other cancer types.

We also considered whether defects in genes commonly mutated in CRC, and known to affect apoptotic pathways, might influence such cell death. Mutation status for APC, β-catenin, p53 and DNA MMR genes (Table 1) does not appear to influence aspirin-induced apoptosis in CRC lines, emphasising the relevance of the aspirin NFκB anti-tumour effect to CRC in general.

Differential sensitivity to the apoptotic effects of aspirin is paralleled by differing responses of the NFκB pathway

Our previous work indicates that NFκB nuclear translocation is a key component of aspirin-induced apoptosis in CRC cells. We therefore considered whether the variations in cell viability, observed between CRC and non-CRC cell lines, were attributable to differing responses of the NFκB pathway to aspirin. We first investigated the effect of aspirin on cytoplasmic levels of the NFκB inhibitor protein IκBα, using immunoblot analysis. We found that aspirin treatment resulted in concentration-dependent degradation of IκBα, as indicated by a reduction in cytoplasmic IκBα protein levels (Figure 2A) in all CRC cell lines. Although the IκBα degradation may be more obvious at the higher doses, there is degradation at the lower doses of 1 and 3 mM aspirin (Figure 2A). The IC_{50} values for the CRC cell lines range from 1.48 to 3.12 mM aspirin, demonstrating differential sensitivity to aspirin with respect to the concentration at which 50% of the cells are growth inhibited (Table 2). The SW480 and HT-29 cell lines have IC_{50} values at the lower end of the range (1.48 and 1.98 mM, respectively) and do undergo IκBα degradation at 1 mM, whereas the HRT-18 and DLD-1 cell lines have IC_{50} values of 3.12 and 2.92 mM and also exhibit IκBα degradation at 3 mM. Hence, there is a close relationship between IC_{50} values and IκBα degradation for the individual CRC cell lines. In striking contrast, there was no change in IκBα levels upon aspirin treatment in any of the non-CRC cell lines even at the highest dose of 10 mM (Figure 2B).

Since these findings suggested a cell-type specific NFκB response to aspirin, we next determined whether the disparate IκBα response was accompanied by a differential effect on NFκB nuclear translocation in the CRC compared to the non-CRC cell lines. Immunofluorescence analysis showed that p65, the transcriptionally active subunit of NFκB, was primarily located in the cytoplasm in untreated cells as expected (Figure 2C, D, first panel). Following aspirin treatment, there was nuclear accumulation of p65 in all of the CRC cells (Figure 2C, second panel). However, in keeping with our observation that there was no IκBα degradation in the non-CRC cells, aspirin treatment did not induce nuclear translocation of p65 in any of these cell lines (Figure 2D, second panel). These data establish that the disparity in viability following exposure to aspirin in CRC lines compared to lines derived from other cancer types is associated with markedly differing responses of the NFκB pathway to aspirin. This work suggests that the effect of aspirin on NFκB signalling may be implicated in the differential sensitivity of cancer types to aspirin-induced apoptosis.

Basal IκBα and p65 protein levels and aspirin-induced apoptosis in CRC cell lines

High basal NFκB activity and aberrant IκBα expression have been observed in a number of cancers including CRC (Rayet and Gelinas, 1999). In view of our findings of a cell-type specific NFκB and death response to aspirin, we considered whether the basal levels of IκBα and p65 might determine increased sensitivity to apoptosis, and so could be potential molecular markers of response. We used immunoblot analysis of cytoplasmic extracts to examine basal levels of IκBα and p65 in both the CRC and non-CRC cell lines (Figure 3). There was no difference in expression of IκBα or p65 or their relative levels (analysed by densitometry, data not shown) between colorectal and non-CRC cells that could account for increased sensitivity to apoptosis. These results indicate that sensitivity to aspirin-induced apoptosis is not related to the cytoplasmic pool of either protein available for stimulation.

Basal COX-2 protein levels do not determine the NFκB response to aspirin

Increased COX-2 expression has been observed both in premalignant colonic lesions and CRCs (Eberhart *et al*, 1994), and COX-2 inhibition has been shown to play a role in aspirin-mediated cell death (Boolbol *et al*, 1996). Hence, we considered whether COX-2 expression might explain the heterogeneity of the aspirin response between the CRC and non-CRC cell lines. Immunoblot analysis of

cytoplasmic proteins demonstrated considerable variation in basal levels of COX-2 between the CRC cell lines (Figure 4). The CRC cell lines SW480 and HCT116 do not express COX-2, whereas HT-29 and LoVo do express COX-2, and yet all underwent apoptosis following aspirin treatment. In the non-CRC panel, the MCF-7 cell line does not express COX-2 but the MDA-MB-231 cell line does

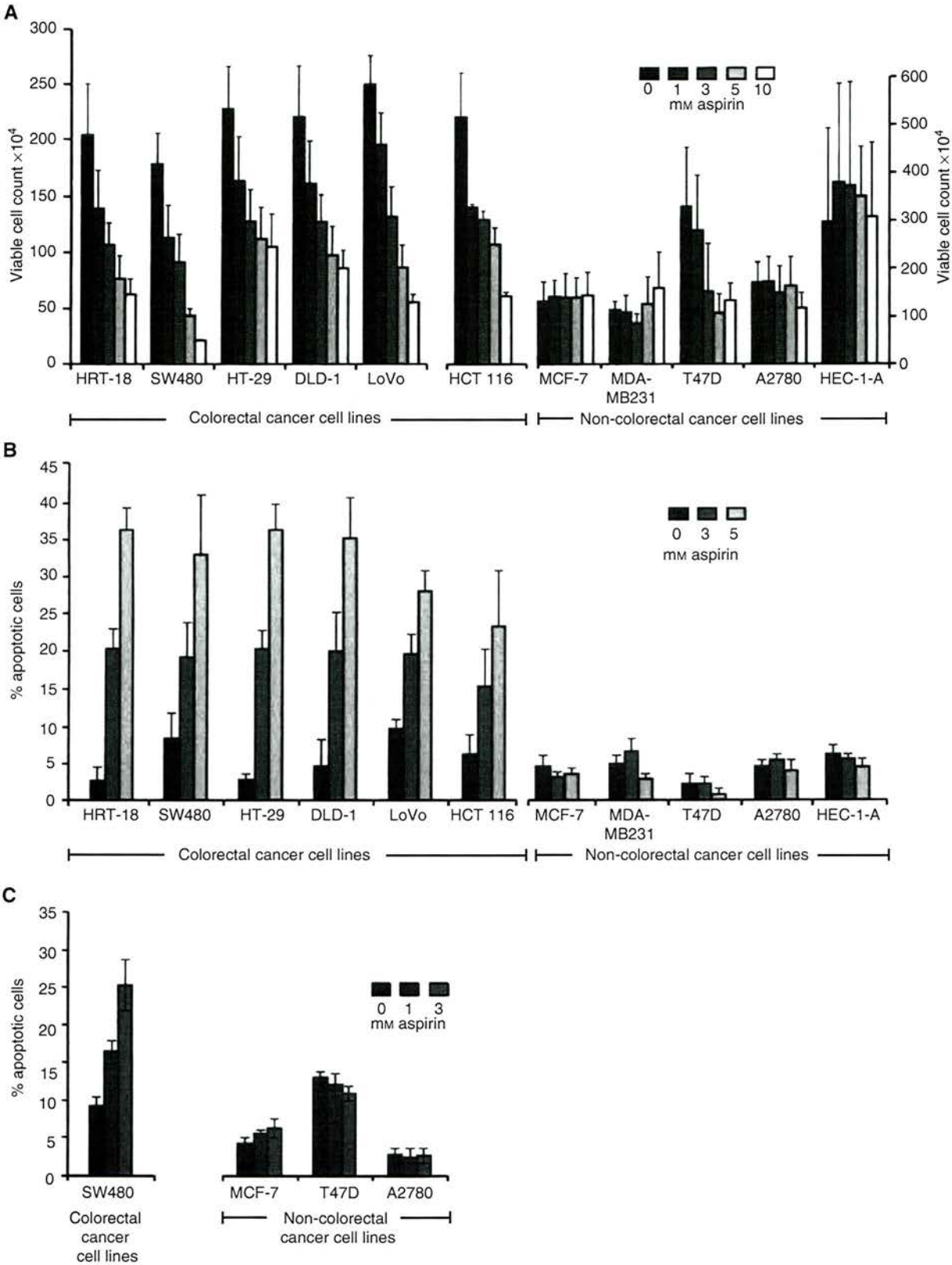

Table 2 IC_{50} values for colorectal cancer cell lines

Cell line	IC_{50}
HRT-18	3.12±0.69
SW480	1.48±0.12
HT-29	1.98±0.68
DLD-1	2.92±0.58
LoVo	2.07±0.25
HCT-116	2.71±0.46

express COX-2, but neither undergoes aspirin-induced apoptosis. Similarly, there was variability of COX-2 levels between the CRC lines and the non-CRC lines (Figure 4). Thus, we found no association between basal levels of COX-2 expression and sensitivity to aspirin-induced apoptosis, providing further support for the notion that COX-independent mechanisms play an important role in the anti-tumour effect of NSAIDs.

DISCUSSION

The work presented here demonstrates a striking difference in the response to aspirin between CRC cell lines and lines derived from other cancer types, with respect to both cell viability and NFκB signalling. We show that aspirin-induced apoptosis, associated with IκBα degradation and NFκB nuclear translocation, was restricted to CRC cells. This relationship between aspirin-induced apoptosis and the effect on NFκB signalling suggests a molecular rationale for the particular sensitivity of CRC to NSAIDs compared to other cancers. These findings also extend our previous observations on the importance of the NFκB pathway as a key NSAID target.

Epidemiological evidence indicates that NSAIDs impart greater protection against CRC than other cancer types, but the molecular basis for this effect is not known. Several previous reports, including our own, have shown that aspirin induces apoptosis in CRC cells (Hanif *et al*, 1996; Elder *et al*, 1997; Piazza *et al*, 1997; Qiao *et al*, 1998; Castano *et al*, 1999; Stark *et al*, 2001). There is little data directly comparing the anti-tumour effects of NSAIDs *in vitro* between CRC cells and cancer cells of different tissue origin. A recent study has demonstrated a tissue type-independent effect in prostate, lung, colon, tongue and pancreatic cancer using nitric oxide-donating NSAIDs and, although treatment with conventional NSAIDs did have a growth-inhibitory effect, it was observed at concentrations in excess of the pharmacologically relevant range after 48 h of treatment (Kashfi *et al*, 2002). Our findings demonstrate that aspirin has a considerable degree of specificity of apoptotic effect for CRC cells compared to other cell lines studied, and this reflects the epidemiological observations in the respective tumours. We show that aspirin induces apoptosis in a panel of CRC cell lines, but has no consistent effect on viability and apoptosis in cancer cell lines of non-colorectal origin. These results contrast with some previous reports of NSAID-induced growth inhibition and apoptosis in breast and endometrial cancer cells (Noguchi *et al*, 1995; Planchon *et al*, 1995; Han *et al*, 1998; Arango *et al*, 2001), but these differences are reconciled by considering that these studies used NSAIDs other than aspirin (Noguchi *et al*, 1995; Planchon *et al*, 1995; Han *et al*, 1998), while others only observed apoptosis after long exposures (48–96 h) to high concentrations of salicylate out with the therapeutic range (Sotiriou *et al*, 1999; Arango *et al*, 2001). The non-CRC cell lines are susceptible to other apoptosis-inducing agents and NFκB activators such as staurosporine and TNFα, respectively, indicating that these cell lines are not generally resistant to apoptosis or NFκB modulation (Mooney *et al*, 2002; Tang *et al*, 2002). The observation that aspirin decreased cell viability in one of the three breast cancer cell lines (T47D) is in keeping with epidemiological data that suggest a lesser protective effect of NSAIDs against breast cancer. One cohort epidemiological study showed that the effect of aspirin use on CRC incidence was reduced in females (Schreinemachers and Everson, 1994), raising the possibility that differential protection may be related to gender. However, gender is unlikely to impart a predominant protective effect, as it has not been borne out by subsequent studies (Thun *et al*, 1993). Indeed, the HT-29 CRC cell line is derived from a female patient and is equally susceptible to aspirin-mediated apoptosis and NFκB modulation as the other CRC cell lines, which are male in origin. Our findings clearly indicate important differences between CRC and other cancer types with respect to aspirin effects on cell viability and apoptosis.

We show that aspirin-induced apoptosis occurs following IκBα degradation and NFκB nuclear translocation, and that this effect is common to all CRC cell lines studied. Notably, this effect on the NFκB pathway was consistent between CRC cell lines despite heterogeneity of the lines, with respect to the profile of mutations in APC, β-catenin, p53 and DNA MMR genes (see Table 1). In contrast, aspirin treatment did not induce IκBα degradation or NFκB nuclear translocation in any cell lines derived from cancers of other tissue types, paralleling the lack of consistent changes in cell viability and apoptosis in these lines. We have previously established that the observed effect of aspirin on IκBα and p65 is a *cause* of rather than a consequence of apoptosis, based on the findings that the IκBα degradation was signal-specific and that nuclear translocation of NFκB and apoptosis were blocked by a dominant-negative super repressor IκBα (Stark *et al*, 2001). Furthermore, we showed that IκB degradation and NFκB nuclear translocation occur at 2–5 h after aspirin treatment and persists thereafter to 24 h, whereas apoptosis is not observed to increase until at least 16 h and continues to 24 h. Hence, the weight of evidence presented here correlating IκBα degradation and p65 nuclear translocation with apoptosis compared to the lack of response in non-CRC cell lines provides considerable further support for a causal role of the NFκB response as an important component of aspirin-induced apoptosis.

Having shown a striking difference between the CRC and non-CRC cell lines, with respect to aspirin effects on NFκB signalling and apoptosis, we investigated potential factors that might contribute to the ability of specific cell types to undergo apoptosis. Increased NFκB activity has been observed in CRC (Hardwick *et al*, 2001) and relative resistance to apoptosis has been attributed to high constitutive NFκB activity in other cancers (Bours *et al*, 1994; Lind *et al*, 2001; Charalambous *et al*, 2003). However, we found no evidence that the specificity of the aspirin-NFκB response is related to differential expression of basal IκBα or p65 proteins or their relative expression. The SW480 and HT-29 CRC cell lines undergo NFκB-mediated apoptosis, despite the considerable

Figure 1 Differential effect of aspirin on cell viability and apoptosis in CRC and non-CRC cell lines. Aspirin treatment (0–10 mM) for 24 h induces a concentration-dependent decrease in viable cell number (determined by haemocytometric counts) in all CRC cell lines, but there is no consistent change in the non-colorectal cancer cell lines (**A**). Annexin V binding assay is used to determine whether all CRC cell lines undergo apoptosis after aspirin treatment (0–5 mM) for 24 h, but there was no change in apoptosis in the non-CRC cell lines (**B**). Annexin V binding assay is used to determine whether the non-CRC cell lines are less susceptible to aspirin-induced apoptosis compared to the CRC cell line SW480 following treatment for 72 h with aspirin (0–3 mM) (**C**). The graphs represent three independent experiments and the bars on the graphs are standard error bars.

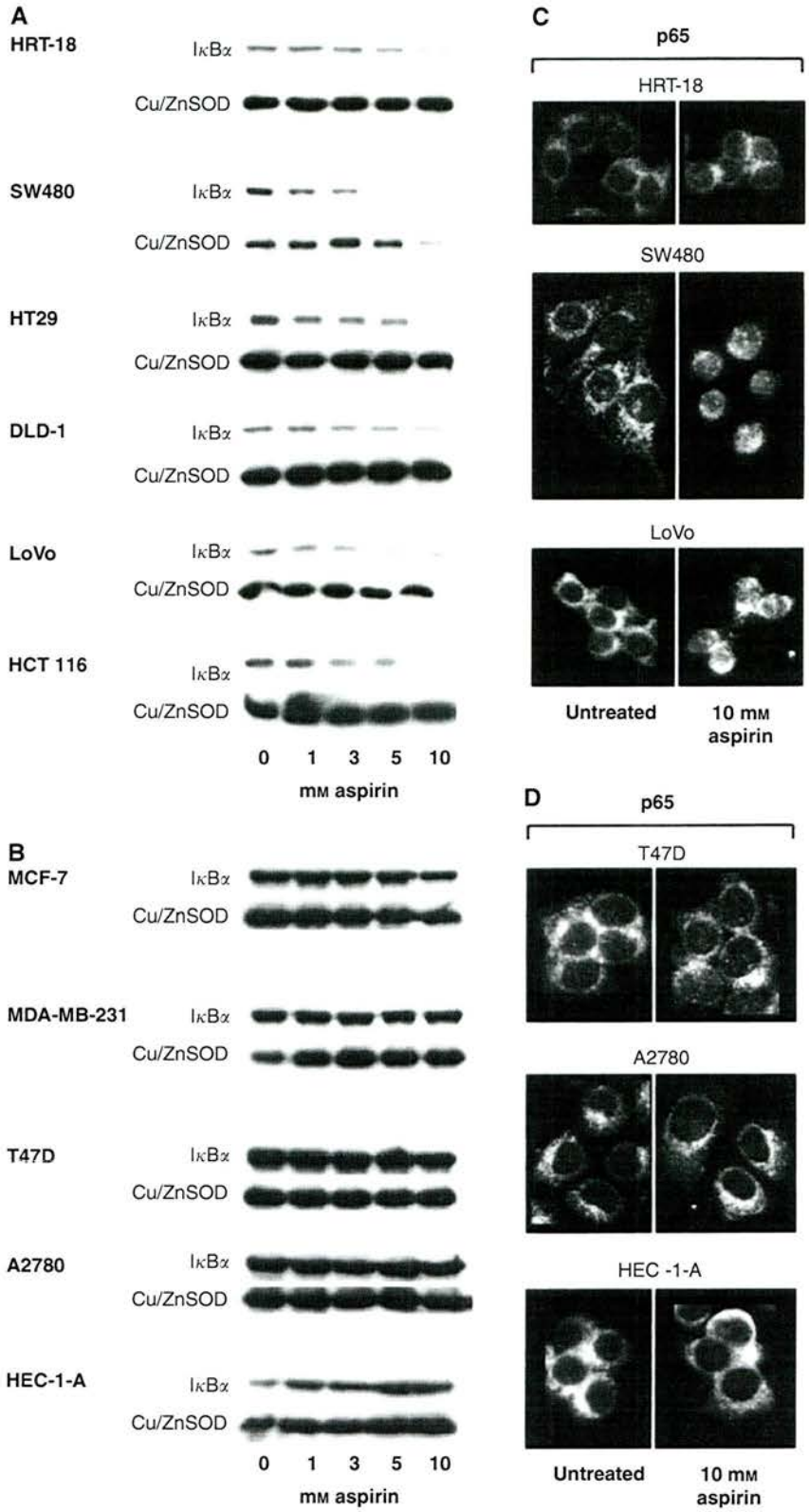

Figure 2 Aspirin-induced IκBα degradation and p65 nuclear translocation is restricted to CRC lines. Western blot analysis shows that aspirin treatment (0–10 mM) for 24 h induces IκBα degradation in a concentration-dependent manner in the CRC cell lines (**A**), but not in the non-CRC cell lines (**B**). Following aspirin treatment, cytoplasmic extracts were made from untreated and treated cells and probed with sheep polyclonal IκBα antibody. The western blot shown is representative of at least three independent experiments, and Cu/Zn SOD was used as a control for protein loading. Micrographs (× 63) of immunocytochemically stained cells show that aspirin treatment (10 mM) for 24 h induces nuclear accumulation of p65 in the CRC cell lines (**C**), but not in the non-CRC cell lines (**D**).

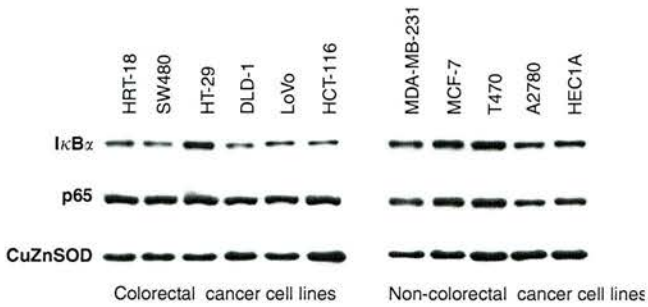

Figure 3 Western blot analysis demonstrates basal expression levels of cytoplasmic IκBα and p65 proteins in CRC and non-CRC cell lines in untreated cells. Cytoplasmic extracts were made from untreated cells and probed with sheep polyclonal IκBα antibody and rabbit polyclonal p65 antibody. The western blot shown is representative of at least three independent experiments and Cu/Zn SOD was used as a control for protein loading.

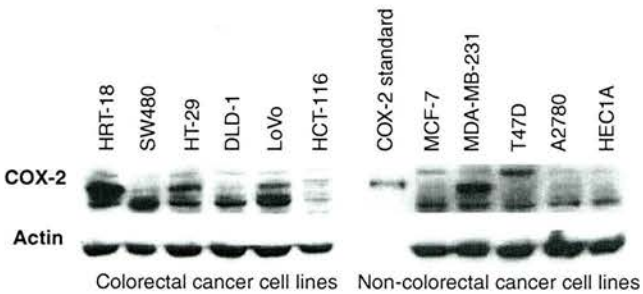

Figure 4 Western blot analysis of basal expression levels of cytoplasmic COX-2 protein in CRC and non-CRC cell lines. Cytoplasmic extracts were made from untreated cells and probed with mouse monoclonal COX-2 antibody. A COX-2 electrophoresis standard (72 kDa monomer) is used to indicate the COX-2 band. COX-2 is expressed in the HRT-18, HT-29, LoVo and MDA-MB-231 cell lines. The Western blot shown is representative of at least three independent experiments and actin was used as a control for protein loading.

difference in basal NFκB activity previously reported between these cell lines (Dejardin *et al*, 1999).

There is substantial rationale for investigating COX-2 as a potential molecular determinant of response, in view of its role in CRC development and as a pharmacological target for NSAIDs. Additionally, it has been reported that the inconclusive nature of epidemiological data in breast cancer might be related to the observation that only a subset of breast cancers express COX-2 (Howe *et al*, 2001). In the work presented here, we did not detect a relationship between COX-2 protein levels and apoptotic response to aspirin in any cell type. Furthermore, the fact that we observed considerable variation in COX-2 expression within the CRC cell lines, which were all susceptible to aspirin-induced apoptosis, presents persuasive evidence that COX-2-independent as well as COX-2-dependent mechanisms play a role in the anti-tumour effects of NSAIDs (Rigas and Shiff, 2000).

Aspirin concentrations used here are relevant to pharmacological levels in clinical practice (1–3 mM) (Insel, 1996). Nonetheless, comparisons between cell culture concentrations and plasma levels are somewhat artificial, because of the inability to accurately mimic *in vivo* metabolism and tissue concentration of the agent in epithelial or tumour cells. Decreased basal levels of apoptosis and hyperproliferative mucosa have been observed in patients with adenomas, suggesting the existence of a 'field defect' in the colonic mucosa (Anti *et al*, 2001). Although we observed proportionally less apoptosis at lower concentrations of aspirin, there is evidence that low levels of apoptosis translate into significant tumour regression over time in cell kinetics studies (Pritchard and Watson, 1996). It remains to be determined whether aspirin redresses the balance by inducing apoptosis *de novo* in newly transformed colorectal epithelial cells destined to become malignant clones. There is evidence of NFκB involvement in colonic crypt differentiation and cell turnover in mouse colon, where NFκB activity is greater in proliferating cells at the base of crypts compared to mature cells at the surface (Inan *et al*, 2000). Thus, it is also possible that the drug corrects deranged mechanisms that permit escape from normal cellular turnover and apoptosis.

In summary, the data presented here demonstrate that there are substantial differences in the anti-tumour effects of aspirin and modulation of NFκB signalling between cancer cells of different tissue origin. The effect of aspirin on NFκB signalling and apoptosis does not appear to be related to expression levels of COX-2 or mutation status of APC, β-catenin, p53 and DNA MMR genes. This is important when considering translating these findings to clinical studies aimed at defining the NFκB response to aspirin in human colonic epithelium and tumours. The molecular basis of NSAID anti-tumour activity is complex, and our findings provide further evidence that the effects of aspirin on NFκB signalling have particular relevance to CRC chemoprevention.

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Aspirin-induced nuclear translocation of NFκB and apoptosis in colorectal cancer is independent of p53 status and DNA mismatch repair proficiency

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Substantial evidence indicates nonsteroidal anti-inflammatory drugs (NSAIDs) protect against colorectal cancer (CRC). However, the molecular basis for this anti-tumour activity has not been fully elucidated. We previously reported that aspirin induces signal-specific IκBα degradation followed by NFκB nuclear translocation in CRC cells, and that this mechanism contributes substantially to aspirin-induced apoptosis. We have also reported the relative specificity of this aspirin-induced NFκB-dependent apoptotic effect for CRC cells, in comparison to other cancer cell types. It is now important to establish whether there is heterogeneity within CRC, with respect to the effects of aspirin on the NFκB pathway and apoptosis. p53 signalling and DNA mismatch repair (MMR) are known to be deranged in CRC and have been reported as potential molecular targets for the anti-tumour activity of NSAIDs. Furthermore, both p53 and MMR dysfunction have been shown to confer resistance to chemotherapeutic agents. Here, we set out to determine the p53 and hMLH1 dependency of the effects of aspirin on NFκB signalling and apoptosis in CRC. We specifically compared the effects of aspirin treatment on cell viability, apoptosis and NFκB signalling in an HCT-116 CRC cell line with the p53 gene homozygously disrupted (HCT-116$^{p53-/-}$) and an HCT-116 cell line rendered MMR proficient by chromosomal transfer (HCT-116^{+ch3}), to the parental HCT-116 CRC cell line. We found that aspirin treatment induced apoptosis following IκBα degradation, NFκB nuclear translocation and repression of NFκB-driven transcription, irrespective of p53 and DNA MMR status. These findings are relevant for design of both novel chemopreventative agents and chemoprevention trials in CRC.
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Ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to be associated with a 40–50% reduction in relative risk of colorectal cancer (CRC) (Thun *et al*, 1993). There is much research effort focussed on the molecular mechanisms involved in NSAID anti-tumour activity. Such understanding would inform the rational design of novel agents both for chemoprevention and therapy.

We previously reported the pivotal involvement of the transcription factor NFκB (p50–p65) in aspirin-mediated apoptosis in CRC cells (Stark *et al*, 2001). NFκB is sequestered in the cytoplasm, bound to a member of the IκB family of inhibitory proteins. Stimulation by one of a range of signals results in phosphorylation and ubiquitination of IκB followed by proteosome-mediated degradation. Dissociation from IκB results in NFκB nuclear translocation and transcriptional regulation of numerous target genes. NFκB has been shown to have both pro- and anti-apoptotic effects (Barkett and Gilmore, 1999). Such disparate effects are due to differences in stimuli, NFκB composition, cell type and distinct κB binding specificities of individual complexes resulting in diverse target gene specificity (Epinat and Gilmore, 1999). We have previously shown that aspirin-mediated apoptosis in CRC cells involves IκBα degradation and NFκB nuclear translocation (Stark *et al*, 2001). We also demonstrated that aspirin-induced IκBα degradation was required for apoptosis because cells constitutively expressing super-repressor IκBα were resistant to both aspirin-induced NFκB nuclear translocation and apoptosis. More recently, we have reported the relative specificity of this NFκB-dependent apoptotic effect of aspirin for CRC cells, when compared to other cancer cell types (Din *et al*, 2004). However, it is also important to determine whether there is heterogeneity within CRC, with respect to the effects of aspirin on the NFκB pathway and apoptosis.

It is now well established that genomic instability, by increasing mutational load, promotes neoplastic progression in CRC. The p53 tumour suppressor gene is involved in cell cycle control, apoptosis and maintenance of genomic stability and is frequently mutated in colorectal tumours, heralding malignant transformation (Baker *et al*, 1989, 1990; Honma *et al*, 2000). Another important contributor to genomic instability is defective DNA mismatch

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repair (MMR) resulting in microsatellite instability (MSI) (Veigl *et al*, 1998). MSI is the hallmark of tumours arising in hereditary nonpolyposis colorectal cancer (HNPCC) and is also found in 15% of sporadic CRCs (Boland *et al*, 1998; Brown *et al*, 1998). The majority of MSI tumours in familial cases are due to germline mutations in hMLH1, hMLH2 and hMSH6 genes, and to hMLH1 promoter hypermethylation in sporadic cancers (Kuismanen *et al*, 2000). p53 dysfunction and DNA MMR deficiency are almost wholly mutually exclusive (Cottu *et al*, 1996; Samowitz *et al*, 2001). p53 signalling and DNA MMR have been identified as potential molecular targets for NSAIDs (Ruschoff *et al*, 1998; Shao *et al*, 2000; Goel *et al*, 2003), suggesting that the anti-tumour effect may, in part, involve countering the effects of genetic instability in CRC. Such genetic aberrations in tumours have also been shown to be involved in determining response to chemotherapeutic agents (O'Connor *et al*, 1997; Weller, 1998; Ribic *et al*, 2003).

In light of the importance of p53 and MMR in CRC and that genomic instability can influence response to chemotherapeutics, we set out to determine the p53 and hMLH1 dependency of the effects of aspirin on NFκB signalling. Our findings suggest that the NFκB-apoptotic response to aspirin occurs irrespective of p53 status and MMR defects and shed further light on the chemopreventive action of NSAIDs.

MATERIALS AND METHODS

Cell line culture and treatment

The CRC cell line HCT-116 (genotype p53 (+/+), hMLH1 (−)), is available from the American Type Culture Collection (ATCC). The HCT-116 cell line has a hemizygous mutation in hMLH1 resulting in a truncated, nonfunctional protein. The HCT-116 subline where hMLH1 expression is restored by chromosome 3 transfer ($HCT116^{+ch3}$, genotype p53 (+/+), hMLH1 (+)) was a gift from Professor CR Boland and these cells are competent in DNA MMR (Koi *et al*, 1994). The p53 null HCT-116 subline ($HCT116^{p53-/-}$) was created by targeted homologous recombination (Bunz *et al*, 1999) and was a gift from Professor B Vogelstein. All three cell lines were grown in McCoy's 5A media and the $HCT116^{+ch3}$ cell line was grown under selection with 0.4 mg ml^{-1} geneticin. All media were supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (media supplied by Gibco BRL, Paisley, UK) and cells were grown as monolayers (37°C in 5% CO_2). Cells were plated (1×10^6 cells/50 ml flask) and grown until 60–70% confluent. Aspirin (Sigma, St Louis, USA) was prepared as a 0.5 M stock solution in distilled water (final pH 7.0). Prior to treatment for 16 h with aspirin (1, 3, 5 and 10 mM) or carrier control (at same concentrations as aspirin), the growth medium was replaced with the respective low serum (0.5% FCS) medium.

Cell viability and determination of apoptosis

After aspirin treatment, adherent cells were harvested and viable cell number determined by counting with a haemocytometer. Cell surface phosphatidylserine is a marker for apoptosis and was detected via its interaction with annexin V using the Annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA, USA), as per the manufacturer's instructions. Briefly, the media from the flask of adherent cells was transferred to a conical tube on ice to harvest any floating cells. Cells were then washed with 2 ml of PBS, which was also added to the tube to collect any cells dislodged during washing. Cells were incubated with 1 ml of trypsin : versene (volume per volume) just until the cells detached and then resuspended in the conical tube containing the media with the floating and washed cells. Cells were counted using a haemocytometer and resuspended in cold 1 × binding buffer to approximately 1×10^6 cells ml^{-1}. Media binding reagent (10 μl) was added to 0.5 ml of the cell suspension, which was incubated with 1.25 μl of annexin V-FITC for 15 min at room temperature in the dark. Annexin V was then removed by centrifugation at 1000 × g for 5 min and the cells were resuspended in 0.5 ml of cold 1 × binding buffer and placed on ice. The counting was carried out using a haemocytometer (two counting grids) in duplicate and this was carried out immediately following staining of cells as apoptosis is an ongoing process and the FITC signal may be lost after an hour.

Western blotting

After aspirin treatment, cells were washed with PBS, centrifuged (1200 r.p.m. for 10 min) and cell pellets were resuspended in lysis buffer (50 mM NCl, 10 mM HEPES, 500 mM sucrose, 1 mM EDTA, 0.5 mM. spermidine, 0.15 mM spermine, 0.2% Triton X-100, Complete Protease Inhibitor Cocktail (Roche Diagnostics, Manheim, Germany), 100 mM Pefabloc (Roche Diagnostics, Manheim, Germany)). The cell suspension was centrifuged (6000 r.p.m. for 15 min, 4°C) and the supernatant used as cytoplasmic extract. Protein content was measured by the method of Bradford (BioRad, Hercules, CA, USA).

Cytoplasmic proteins (30 μg) were separated on a 10% SDS–PAGE gel, transferred to a polyvinylidine difluoride membrane (BioRad) and blocked in 4% non-fat dry milk solution with 0.3% Tween20 (Sigma). Membranes were probed with a sheep polyclonal IκBα antibody (a gift from Professor R Hay, University of St Andrews, UK) and then with monoclonal antibody to Cu/Zn SOD (The Binding Site, Birmingham, UK) as a control for protein loading. Chemiluminescence was used to visualise the antigen–antibody complexes (Amersham ECL Reagents, Little Chalfont, UK).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were carried out by incubating nuclear extracts from untreated cells (6 μg) with binding reaction mix (1 × binding buffer (50 mM KCl, 20 mM HEPES, 5% glycerol, 1 mM EDTA, 1 mM DTT), 1 μg BSA, 1 μg poly dI-dC, 25 fmol radioactively labelled oligo-DNA in a final volume of 20 μl) for 30 min prior to analyses on a 4% native polyacrylamide gel. Double-stranded oligonucleotides for NFκB were obtained from Santa Cruz.

Immunofluorescence analysis

Cells grown to 60–70% confluence on glass coverslips were treated with carrier or 10 mM aspirin for 16 h (in respective 0.5% FCS medium). After treatment, cells were washed with PBS, fixed with acetone : methanol (volume per volume) (−20°C, 10 min) and blocked in 10% nonimmune donkey serum (Sigma) for 1 h. Rabbit polyclonal antibody to NFκB p65 (Santa Cruz) was applied for 1 h followed by incubation with FITC-conjugated donkey anti-rabbit IgG for 1 h. The nuclei were stained with DAPI and the coverslips mounted with Vectashield (Vector Laboratories, Burlingame, California, USA).

Transfections and reporter assays

For transient transfection experiments, 50 ml flasks of cells were grown to subconfluency then transfected with 6 μg of luciferase reporter plasmid and 3 μg of β-galactosidase control plasmid, using Lipofectamine as described by the manufacturer's instructions (Gibco BRL). Following transfection, cells were grown in low serum (0.5% FCS) medium then treated with aspirin (0–10 mM) for 16 h. Luciferase activity was measured in cell extracts using a luciferase reporter assay kit (Promega) and read using a luminometer. Transfection efficiency and cell viability were

monitored by cotransfection with a CMV-β-galactosidase reporter plasmid and β-galactosidase activity was quantified with an assay kit (Promega), as per the manufacturer's instructions. Relative luciferase activity was calculated as unit of luciferase activity per unit of β-galactosidase activity.

RESULTS

Basal expression of IκBα and p65 proteins in CRC cell lines is independent of p53 and hMLH1 expression

To investigate the relationship between p53 and MMR status and the NFκB-dependent apoptotic response to aspirin, we used the following three CRC cell lines: parental HCT-116 cells (wild-type p53, hMLH1 deficient), HCT-116^{+ch3} (wild-type p53, hMLH1 proficient) and HCT-116$^{p53-/-}$ (p53 null, hMLH1 deficient). The presence of hMLH1 in the HCT-116^{+ch3} cell line and the absence of p53 in the HCT-116$^{p53-/-}$ cell line was verified by immunoblot analysis (Figure 1A). Firstly, we considered whether p53 and hMLH1 mutation status might affect basal levels of IκBα and p65 proteins. Using immunoblot analysis of cytoplasmic extracts, we found there were no significant differences in expression of IκBα or p65, or their relative levels (data not shown), between the three CRC cell lines (Figure 1B). These results indicate that changes in MMR mutation status and p53 expression do not affect the cytoplasmic pool of either protein available for stimulation. We next considered whether basal levels of NFκB DNA binding were affected by changes in p53 or MMR status. Electrophoretic mobility shift assays (EMSAs) performed on nuclear extracts of untreated cells showed basal NFκB DNA binding in the three CRC cell lines (Figure 1C). The differences in basal levels of NFκB DNA binding complexes in the HCT-116^{+ch3} and HCT-116$^{p53-/-}$ cell lines compared to the parental HCT-116 cell line were marginal (densitometry data not shown).

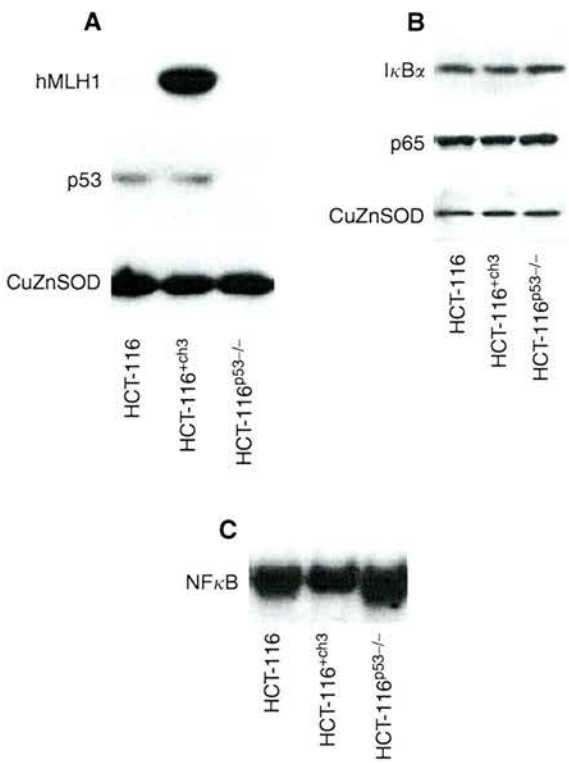

Figure 1 Basal expression of p53, hMLH1, IκBα and p65 in untreated colorectal cancer cell lines. Cytoplasmic extracts were made from untreated cells and Western blots probed with p53 and hMLH1 antibodies to confirm the expression profile of the cell lines (**A**). Cytoplasmic extracts were probed with sheep polyclonal IκBα and rabbit polyclonal p65 antibodies to examine the basal expression of proteins in the cell lines (**B**). The Western blots shown are representative of at least three independent experiments and Cu/Zn SOD was used as a control for protein loading. Electrophoretic mobility shift assay using NFκB consensus oligonucleotide, performed on nuclear extracts of untreated cells, demonstrated basal NFκB DNA binding activity (**C**).

Aspirin induces apoptosis in CRC cell lines independent of p53 and MMR status

To determine whether p53 and MMR status affects the aspirin-mediated reduction in cell viability we have previously observed, we compared the effects of aspirin treatment on the viability of HCT-116^{+ch3} and HCT-116$^{p53-/-}$ cell lines to the parental HCT-116 cell line. In triplicate dose–response experiments, all CRC cell lines were treated for 16 h with aspirin (1, 3, 5 and 10 mM) and viable cell number determined by haemocytometric counts. Aspirin treatment resulted in a concentration-dependent decrease in the number of viable cells in all three CRC cell lines (Figure 2A). Furthermore, the cell lines showed proportionate decreases in cell viability at each concentration increment, indicating a similar sensitivity to aspirin, irrespective of p53 or MMR status (Figure 2B). The IC_{50} values were calculated from the growth curves of the aspirin-treated CRC cell lines (Table 1) and there were no significant differences in levels of cell death with each genetic background.

It is well established that aspirin induces apoptosis in CRC cell lines (Piazza *et al*, 1995; Qiao *et al*, 1998; Stark *et al*, 2001; Din *et al*, 2004). We next confirmed that the reduction in viable cell number in each of the cell lines was indeed due to apoptosis for all three genotypes, and not simply a growth inhibitory effect. Following aspirin treatment, cells were stained with Annexin V, which binds phosphatidylserine residues that are externalised during apoptosis and thus serves as a marker for programmed cell death. Consistent with the reduction in cell viability, we found that aspirin induced a concentration-dependent increase in the proportion of apoptotic cells in all three CRC cell lines (Figure 2C). Furthermore, there was no significant difference in apoptotic response between any of the HCT-116, HCT-116^{+ch3} and HCT-116$^{p53-/-}$ cell lines. In addition there was good correlation between the IC_{50} value and apoptotic response; the lower the IC_{50} value, indicating greater sensitivity at a lower aspirin concentration, the greater the fold increase in apoptosis ($r = -0.98$). These data suggest that aspirin-mediated apoptosis in CRC cells is independent of p53 and MMR status.

Aspirin induces IκBα degradation and NFκB nuclear translocation in CRC cell lines irrespective of p53 and MMR status

Having shown previously that p65 nuclear translocation is a critical event in effecting the apoptotic response to aspirin (Stark *et al*, 2001), we next studied the effect of aspirin on NFκB signalling in each cell line. Since IκBα sequesters NFκB in the cytoplasm, we first investigated the effect of aspirin treatment on the cytoplasmic levels of IκBα in each cell line using immunoblot analysis. We found that aspirin treatment induced degradation of IκBα in a similar concentration-dependent manner in each cell line genotype (Figure 3A). We next determined whether the IκBα degradation was accompanied by NFκB nuclear translocation in the CRC cell lines. Immunofluorescence analysis showed that following 16 h aspirin exposure, p65 translocated from the cytoplasm to the nucleus in each cell line, irrespective of p53 and MMR status (Figure 3B). These findings indicate that aspirin-induced apoptosis, due to modulation of the NFκB pathway,

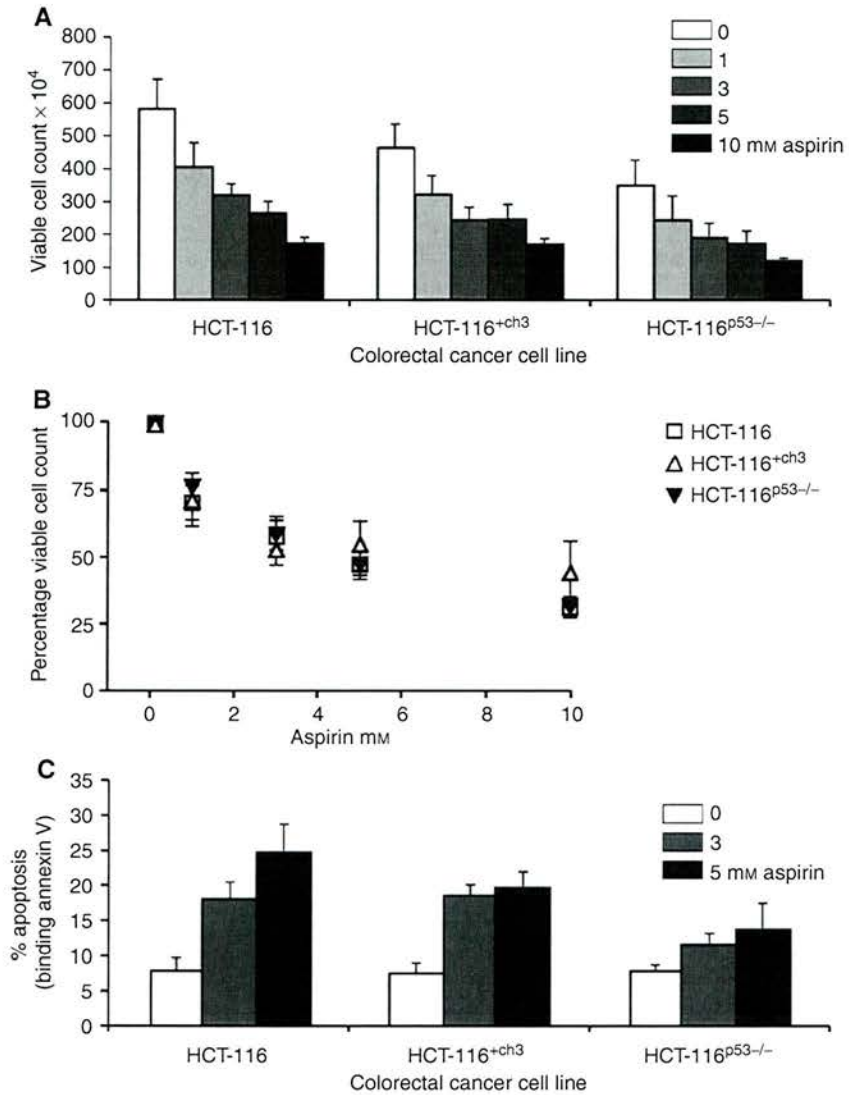

Figure 2 Effect of aspirin on cell viability and apoptosis in colorectal cancer (CRC) cell lines. Aspirin treatment (0–10 mM) for 16 h induces a concentration-dependent decrease in viable cell number (determined by haemocytometric counts) in all CRC cell lines (**A**). The decrease in cell viability is proportionate at each concentration increment indicating a similar pattern of response following aspirin treatment in each cell line (**B**). Annexin V binding assay used to determine that all CRC cell lines undergo apoptosis after aspirin treatment (0–5 mM) (**C**). The graphs represent three independent experiments and the bars on the graphs are standard error bars.

occurs irrespective of derangements in p53 signalling and the MMR system.

Aspirin-mediated repression of NFκB-driven reporter activity is unrelated to p53 and MMR status

We have previously demonstrated aspirin-induced nuclear translocation of NFκB results in repression of NFκB transcriptional activity in CRC cell lines (Stark *et al*, 2000). This was recently substantiated by the finding that NFκB induced by some cytotoxic stimuli acts as an active repressor of anti-apoptotic gene expression (Campbell *et al*, 2004). The effect of aspirin on NFκB-driven transcription was investigated to determine whether p53 or MMR mutation status affects the ability of aspirin to induce NFκB transcriptional repression in CRC cells. The cell lines were transiently transfected with the 3enhancer-ConA NFκB-dependent luciferase reporter construct in which transcription of the firefly luciferase gene is driven by three κB binding sites (Roff *et al*, 1996). A reporter plasmid with deleted κB sites served as control. Following transfection, cells were exposed to aspirin for 16 h. There was a substantial decrease in the basal levels of NFκB-driven reporter activity following aspirin exposure and this was observed in a concentration-dependent manner, irrespective of p53 or MMR status (Figure 4). These findings show that aspirin-induced repression of NFκB transcriptional activity, and hence downstream regulation of target genes, is independent of p53 signalling and DNA MMR.

DISCUSSION

We previously reported that the effects of aspirin on NFκB signalling are a centrally important mechanism of aspirin-mediated apoptosis in CRC cells (Stark *et al*, 2001; Din *et al*,

Table 1 IC_{50} values for colorectal cancer cell lines

CRC cell line	IC_{50}	Fold increase apoptosis	
		3 mM	5 mM
HCT-116	2.8	2.3	3.1
HCT-116^{+ch3}	3.1	2.4	2.6
HCT-116$^{p53-/-}$	4.3	1.5	1.8

Cells were treated with aspirin (1,3,5,10 mM) for 16 h and cell numbers determined as described in Materials and Methods. There is an inverse correlation between IC_{50} values and the fold increase in apoptosis at both 3 and 5 mM ($r = -0.93$ and -0.98). Results are mean of at least three different experiments.

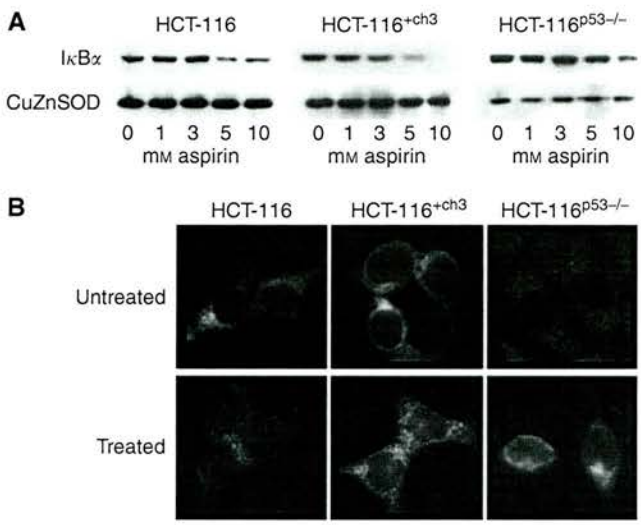

Figure 3 Aspirin induces IκBα degradation and nuclear translocation of p65 in colorectal cancer (CRC) lines. Following aspirin treatment, cytoplasmic extracts were made from untreated and treated cells and probed with sheep polyclonal IκBα antibody. Western blot analysis shows that aspirin treatment (0–10 mM) for 16 h induces IκBα degradation in a concentration-dependent manner in the HCT-116, HCT116^{+ch3} and HCT-116$^{p53-/-}$ CRC cell lines (**A**). The Western blot shown is representative of at least three independent experiments and Cu/Zn SOD was used as a control for protein loading. Micrographs (63 ×) of immunocytochemically stained cells show that aspirin treatment (10 mM) for 16 h induces nuclear accumulation of p65 in the HCT-116, HCT116^{+ch3} and HCT-116$^{p53-/-}$ CRC cell lines (**B**).

2004). This work considerably extends these previous observations since we have shown that the effect of aspirin on apoptosis and NFκB signalling is independent of p53 and DNA MMR status. Furthermore, we show that aspirin induces nuclear translocation of p65 that is associated with repression of κB-driven transcription, again independent of p53 or MMR status.

It is well established that aspirin has a chemopreventive effect in CRC, but the mechanism of action has not been fully characterised. It is apparent that inhibition of the cyclooxygenase-2 enzyme (COX-2) plays a part in the anti-tumour effect of NSAIDs in CRC (Marnett and DuBois, 2002). However, it is also clear that there are other important mechanisms involved since the growth inhibitory and apoptotic effects of NSAIDs occur in CRC cell lines that do not express COX-2 (Hanif *et al*, 1996; Elder *et al*, 1997). Furthermore, NSAIDs that lack COX-2 activity are growth inhibitory and also induce apoptosis in CRC cells (Piazza *et al*, 1995, 1997; Elder *et al*, 1997). It has also been shown that NSAID concentrations required for growth inhibition differ from those for COX-2 enzyme inhibition (Charalambous and O'Brien, 1996; Hanif *et al*, 1996). Taken together, such evidence indicates that while COX-2 inhibition is important, there are other essential mechanism(s) of action. p53 function and DNA MMR have been proposed as potential targets responsible for the anti-tumorigenic properties of NSAIDs (Ruschoff *et al*, 1998; Shao *et al*, 2000).

The p53 signalling pathway is central to regulating cell growth and death, and stabilisation of p53 by mutation is a key event occurring late in colorectal tumorigenesis (Baker *et al*, 1990). Several studies have shown an association between p53 mutation status and sensitivity to chemotherapeutic drugs in colorectal and other cancers (O'Connor *et al*, 1997; Weller, 1998). The p53 pathway has been postulated as a potential target since NSAIDs have been shown to alter levels of p53 (Goldberg *et al*, 1996; Kralj *et al*, 2001). Furthermore, there is evidence for regulatory interdependence between p53 and NFκB involving competition for common coactivators (Wu and Lozano, 1994; Webster and Perkins, 1999). Indeed, wild-type p53 has been shown to suppress constitutive NFκB activity and lead to apoptosis (Shao *et al*, 2000), suggesting that tumours expressing wild-type p53 may be more susceptible to aspirin-induced apoptosis.

Our previous work indicated that CRC cell lines expressing wild-type p53 were not more sensitive to aspirin-induced apoptosis mediated by NFκB signalling, but there are differences other than p53 status between the cell lines studied (Din *et al*, 2004). In this study, we specifically investigated the effects of p53 on the NFκB-induced apoptotic response by use of HCT-116 cells with the p53 gene homozygously disrupted by targeted homologous recombination (Bunz *et al*, 1999). Using this approach, we have shown that aspirin-induced apoptosis is independent of p53 in CRC cells. Furthermore, p53 does not appear to play a role in aspirin-induced effects on NFκB signalling or on the repression of NFκB transcriptional activity. These findings are important in terms of chemoprevention since p53 mutant CRCs have been shown to differ in behaviour from those expressing wild-type p53, with respect to response to chemotherapeutic agents and prognosis.

Defective DNA MMR is characteristic of HNPCC and around 15% of sporadic CRCs also exhibit genetic instability, mainly due to epigenetic silencing of hMLH1 but also to somatic MMR gene defects (Herman *et al*, 1998). The DNA MMR system has been implicated as a potential pathway for modulation that may contribute to NSAID anti-tumour activity (Ruschoff *et al*, 1998; Goel *et al*, 2003). Our previous work suggested that MMR proficient cells may be more sensitive to aspirin-induced apoptosis since the MMR-deficient cell lines had greater IC_{50} values than MMR-proficient cell lines (Din *et al*, 2004). Hence, we examined whether MMR status influenced NFκB-dependent aspirin-induced apoptosis, by comparing the MMR-deficient HCT-116 to its proficient counterpart HCT116^{+ch3}. We observed a dose-dependent increase in apoptosis after treatment with aspirin following IκBα degradation, NFκB nuclear translocation and repression of NFκB-driven transcription in both cell lines. The parental HCT-116 cells, which are MMR deficient, did appear to have a marginally lower IC_{50} value and show a greater fold increase in apoptosis when compared to the MMR-proficient cell line, but this was not significant (Table 1). Although we did not detect any significant differences in the NFκB-dependent apoptotic response to aspirin attributable to MMR status, long-term *in vitro* aspirin exposure has been shown to select for microsatellite stability in colorectal and gastric cancer cell lines (Ruschoff *et al*, 1998; Yamamoto *et al*, 1999). It has also recently been shown that aspirin treatment increased MMR protein expression and apoptosis in CRC cell lines (Goel *et al*, 2003). However, we found no evidence that MMR status influences the NFκB-dependent apoptotic response, suggesting that the MMR system is not the predominant pathway responsible for NSAID anti-tumour activity.

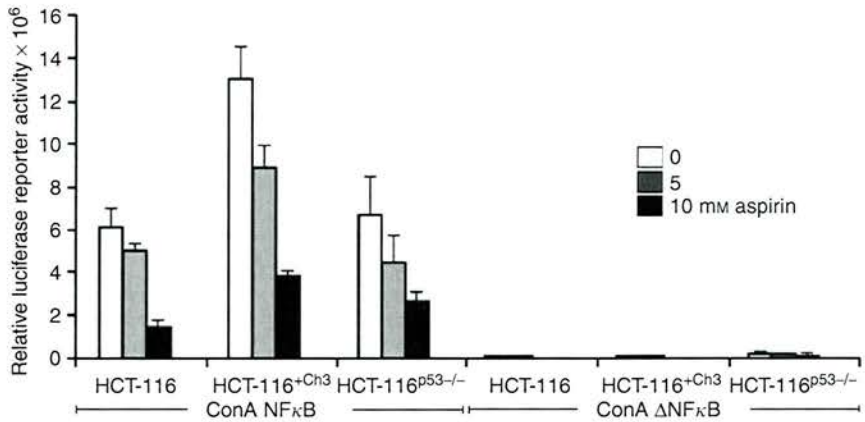

Figure 4 Aspirin induces repression of NFκB-driven transcription in colorectal cancer (CRC) lines. CRC cells were transfected with the ConA NFκB dependent luciferase reporter construct, containing three κB binding sites, or the equivalent plasmid with κB consensus sites deleted (ConA ΔκB). All cells were cotransfected with the control CMV-β-galactosidase plasmid. Following 16 h treatment with 0–10 mM aspirin, luciferase and β-galactosidase assays were performed on cell lysates and relative luciferase activity calculated. The graphs represent three independent experiments and the bars on the graphs are standard error bars.

This work consolidates our previous findings that aspirin-induced apoptosis occurs after IκB degradation, NFκB nuclear translocation and repression of NFκB-driven transcription. The results presented here shed further light on the complex mechanisms by which NSAIDs induce cell death in CRC. Elucidation of the mechanism lies in defining the relative contribution of putative targets to aspirin's anti-tumour activity. We found no evidence for the involvement of p53 or DNA MMR on inducing the NFκB pathway, nor on the ensuing apoptotic response. Genomic instability due to p53 or MMR dysfunction has been to shown to be associated with resistance to chemotherapeutic agents. Hence, these findings may inform rational design of novel therapeutics. In addition, since aspirin effects on NFκB and apoptosis occur in cancers arising from different genetic backgrounds, these findings are clinically relevant when considering design of chemoprevention trials, both in genetically predisposed individuals with defective MMR and in low and moderate risk populations, since p53 mutational events are important during development and progression of colorectal neoplasia.

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